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Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disease (MPD) characterized by the expression of the *p210-BCR/ABL* fusion gene [1]. This gene is produced by the reciprocal translocation (9; 22) (q34; q11) that juxtaposes the 3'end of Abelson leukemia virus (ABL) gene with the 5' end of the breakpoint cluster region (Bcr) gene on chromosome 22. The transcript formed as a result encodes for the BCR/ABL fusion protein with constitutively active tyrosine kinase activity [2-6]. Studies with inducible BCR/ABL transgenic mice showed that expression of BCR/ABL in hematopoietic stem cells and progenitors (HSC/P) is required and is sufficient to induce MPD [7]. If untreated, chronic phase (CP) CML patients progress to a poor-prognosis myeloid or lymphoid blastic phase (BP). The only curative treatment for CML is allogeneic HSC transplantation. The long-term survival rate for this procedure is approximately 65%, however, the procedure is only available to a minority of CML patients due to a lack of compatible donors and age [8-10]. Imatinib is an ABL kinase inhibitor that shows significant activity in CP CML and Ph-positive acute leukemias [11]. By selective induction of apoptosis of BCR/ABL-positive cells [12-14], it provides an effective treatment in CML and has rejuvenated the field of rationalized drug design. The selective inhibitory activity of imatinib toward BCR/ABL has been associated with three problems: the emergence of BCR/ABL mutants in the kinase domain that confer resistance to imatinib; the evidence that CML stem cells are the least vulnerable to ABL-targeted therapy and may serve as reservoirs for occult CML progression; and the relatively low impact of imatinib therapy on the outcome of BP CML patients [15-18]. Resistance to imatinib has an incidence of 4% annually [19]. Different mutated residues have been reported [20], with mutations of Y253, E255, T315, and M351 giving rise to approximately 60% of resistance-conferring mutations in patients at the time of relapse. Efforts to overcome mutant-derived imatinib resistance have led to the development of newer generations of ABL-kinase inhibitors (dasatinib, nilotinib, PD166326, etc.) that are ATP-binding independent. The utility of ABL kinase based rationalized drug design has been demonstrated in recent studies [21-23] that showed that dasatinib and nilotinib are effective against imatinib-resistant BCR/ABL mutants. However, the relatively frequent mutation T315I is insensitive to all the tested tyrosine kinase inhibitors and require alternative therapeutic strategies [24-26]. In addition, many patients present with persistent, BCR/ABL expression in HSC/P [27], also called residual disease and once patients fall into BP CML, their clinical outcome practically remains

unchanged compared with the pre-imatinib era [17]. An alternative to bypass the imatinib-resistance is to target other signaling components downstream of BCR/ABL. During the first year of this award, we reported our progress on the analysis of the role of Rac GTPases in chronic-phase and blastic-phase CML in a retroviral transduction model of leukemia initiation and a novel Rac GTPase inhibitor was shown to impair murine and human leukemogenesis in vitro and in vivo [28] . Since p210-BCR-ABL is also required for leukemia maintenance, we also studied the role of Rac GTPases in an inducible binary murine model of chronic myelogenous leukemia. As a result of this work during the 2nd year of award, three publications have been published in 2008, four oral communications to the International Society of Experimental Hematology and American Society of Hematology were presented in 2008 and two manuscripts are in preparation.

Body

Rac3 is not involved in primary CML initiation.

Unlike Rac2 or the combination of Rac1 and Rac2 (see annual report in 2008), Rac3 deficiency does not impair leukemogenesis initiation in a retroviral transduction model **(Figure 1)**. Although it is possible that Rac3-mediated signaling is involved in blastic transformation, it appears unlikely it is necessary for development of chronic phase CML.

A murine model of CML maintenance has been validated

All our genetic data on the role of Rac GTPases in p210-BCR-ABL induced leukemogenesis were obtained from mice transplanted with BM cells transduced with p210-BCR-ABL. This model is not adequate to answere the question on whether targeting Rac in "real stem cells" will prevent leukemia initiation and/or maintenance in vivo. For that reason, we have invested a large amount of effort in the validation of a murine model of CML maintenance based on the binary expression of two transgenes (SCL-tTA x TRE-BCR/ABL), inducible upon removal of doxycycline (***tet-off***) where expression of p210 BCR-ABL has been restricted to HSC/P cells under the stem cell leukemia (SCL) promoter. The absence of previous data on specific details on leukemic transformation phenotype of stem cells in this model obliged us to generate all these data before asking the question regarding the role of Rac GTPases in this relevant murine model.

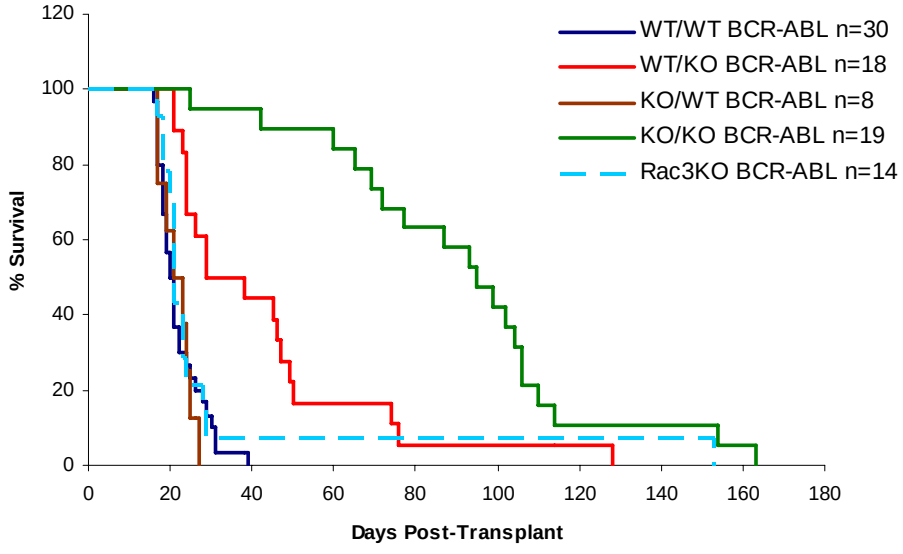


Figure 1. Rac3 does not impair leukemogenesis in a retroviral-transduction model. While Rac2 and especially the deficiency of Rac1 and Rac2 induce a major Impairment of leukemogenesis initiation, Rac3 deficiency does not impair leukemogenic initiation of the murine equivalent of chronic-phase CML.

Our data indicate that unlike the control (DOX-ON) mice induction of p210 BCR-ABL promotes leukocytosis, neutrophilia and significant degree of splenomegaly within 16-18 weeks post withdrawal of the antibiotic doxycycline (DOX-OFF), resembling a myeloproliferative disorder (MPD) **(Figure 2)**. This myeloproliferation is accompanied with activation of CrkL and p38 in splenocytes and

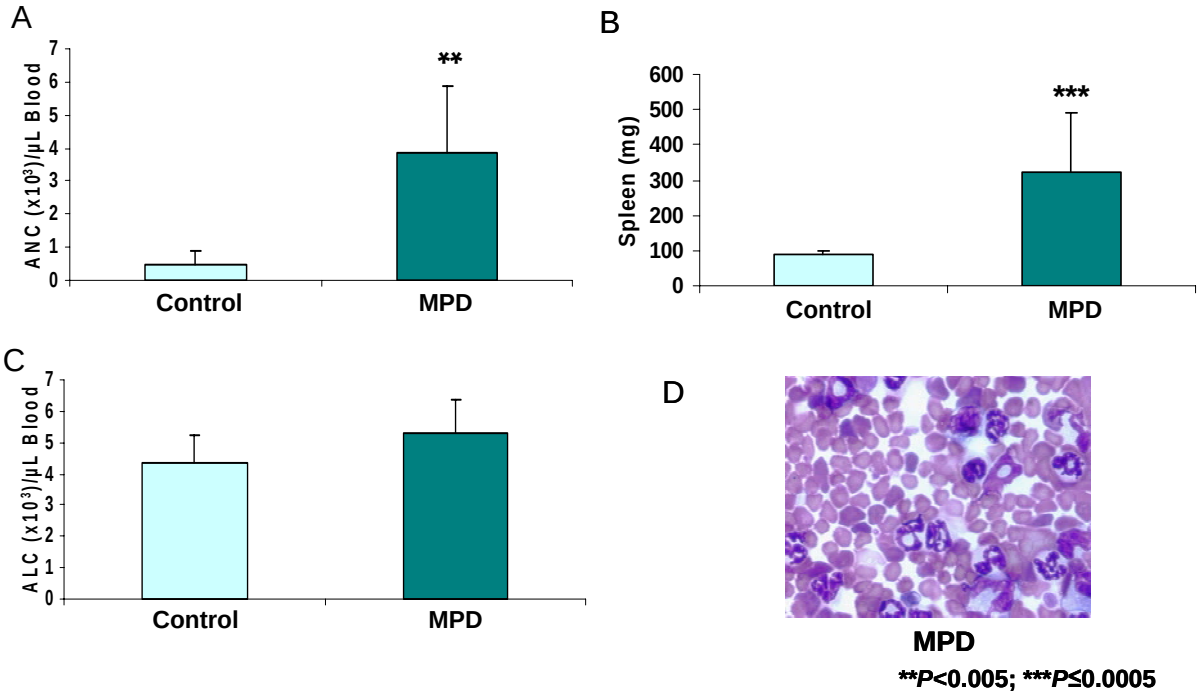


Figure 2. ScltTA x TRE-BCR-ABL mice develop myeloproliferative disease. Double transgenic mice were maintained doxycycline-free for 14-18 weeks. Peripheral blood (A, C, D) and spleens were analyzed. Controls are both non-transgenic, single transgenic mice (Scl-tTA or TRE-BCR-ABL) and double transgenic DOX-ON littermates.

purified bone marrow HSC/P (defined as the Lineage-negative/Sca1+/c-kit+ and Lineage-negative/c-kit+/Sca-1- cell populations) **(Figure 3)**.

CML disease in these mice is transmissible to secondary recipients confirming the stem cell leukemia origin of the disease developed in these mice **(Figure 4).** To further confirm this point, we confirmed the BCR-ABL expression of HSC (Lin^-/$Sca\text{-}1^+$/$c\text{-}kit^+$ cells; LSK) and progenitors (Lin^-/$c\text{-}kit^+$; LK) in these mice **(Figure 5)**. In these mice, leukemia development is associated with activation of downstream signaling effectors CrkL and JNK in leukemic stem cells and progenitors **(Figure 6)**, increased cycling of LSK cells **(Figure 7)**.

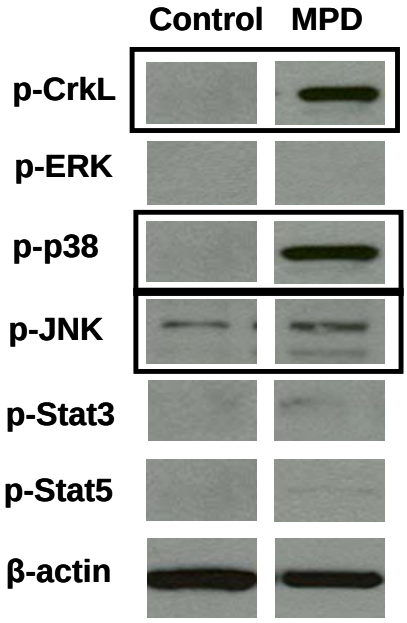


Figure 3. BCR-ABL downstream signaling pathways in leukemic splenocytes are activated.

Interestingly, we could also notice increase in circulating and splenic HSC/P in double-transgenic, DOX-OFF mice, with no correlating increase and a trend to decrease in BM **(Figure 8),** suggesting BM egression, possibly secondary to deregulated homing/engraftment. Homing of leukemic Lineage-negative splenocytes in BM and spleen is increased compared to their control counterparts *in vivo* 16h post-transplantation. This is even more interesting when we also see that the homing of lineage-negative BM cells from DOX-OFF mice in spleen, but not in BM, is also increased **(Figure 9A)** suggesting that, in this model, leukemic HSC/P mostly home in spleen but not in BM, in agreement with our working hypothesis. These data are strengthened by the finding that the spleens of leukemic mice are enriched, while their BM is depleted, in HSC/P which can locate in the BM endosteum **(Figures 9B-C).** Finally, leukemic BM stem

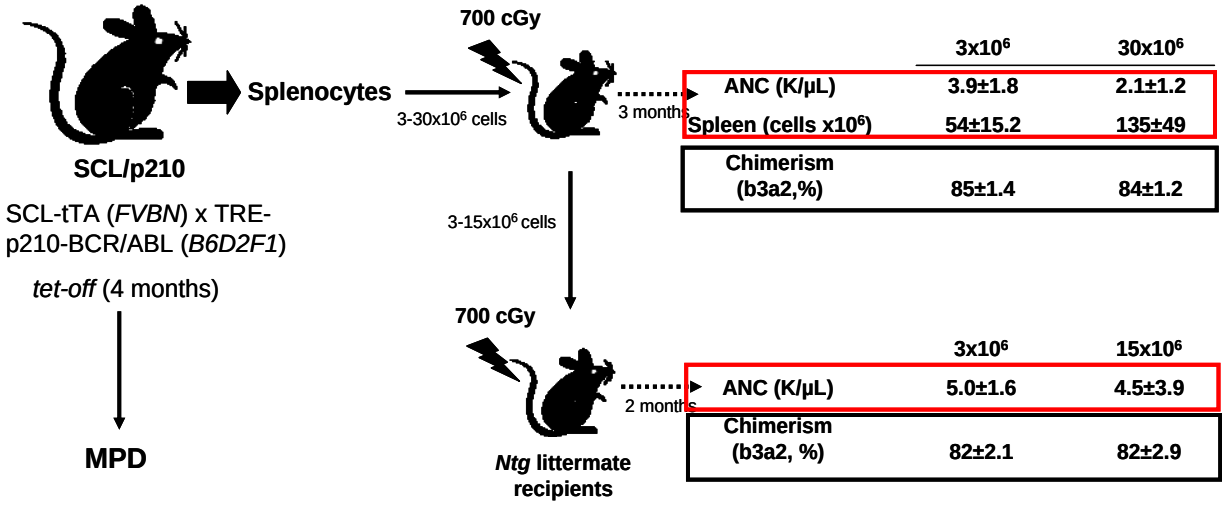


Figure 4. Splenocytes from primary MPD mice can transfer the disease to non-transgenic littermate secondary recipients. Secondary recipients received sublethal irradiation (7Gy) and 3 to 30x10^6 splenocytes. Secondary recipients developed MPD in 2 months. Tertiary recipients received 3 to 15x10^6 splenocytes and developed similar disease in 2 months.

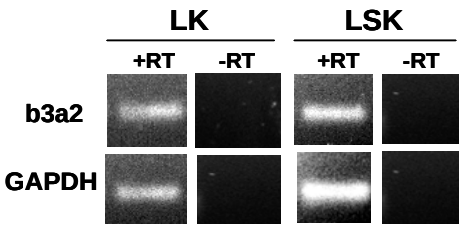


Figure 5. BCR-ABL (b3a2) expression in leukemic Progenitor (LK) and stem cell (LSK) compartments.
RT=reverse transcriptase.

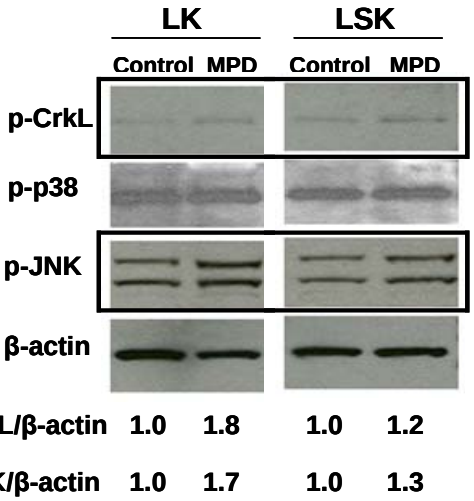


Figure 6. BCR-ABL downstream signaling pathways in leukemic progenitors (LK) and stem cells (LSK) splenocytes are activated.

cells and progenitors show increased CD44 expression and decreased L-selectin expression on their membrane, similar to what has been shown in human CML **(Figure 10A-B)** and hematopoietic progenitors show less adhesion to fibronectin and increased migration towards stromal cell-derived factor (SDF1-α) compared to control cells **(Figure 10C-D)**.

Altogether, this murine model represents an adequate *in vivo* system to analyze LSC/P intrinsic transformation events affecting homing, migration and proliferation.

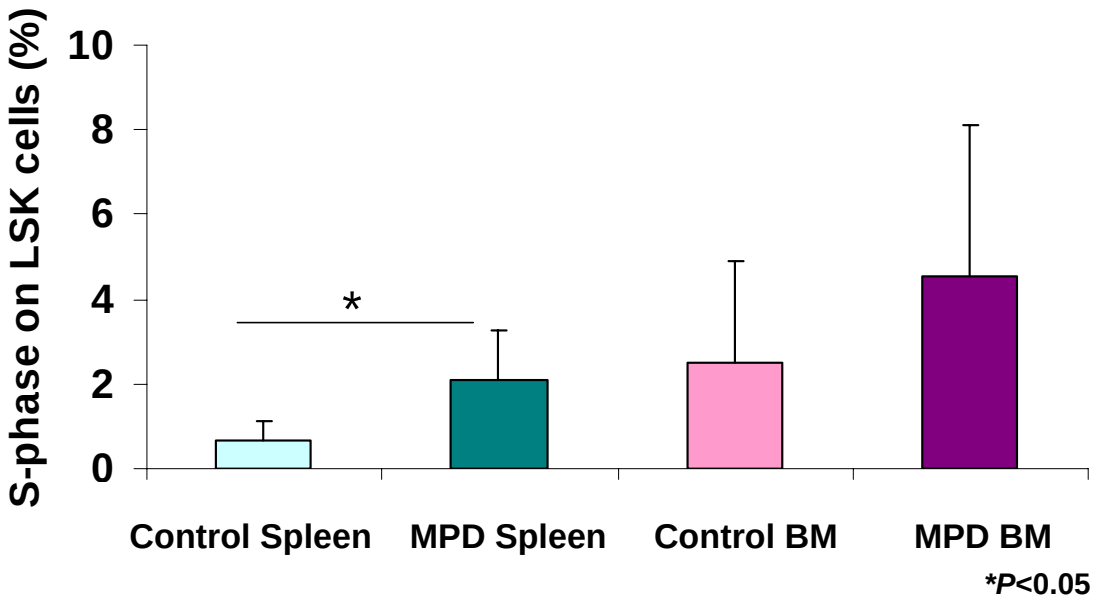


Figure 7. Proliferation of HSC/P (Lin-/c-kit+/Sca-1+) in spleen and BM of control (non-transgenic or dox-on transgenic mice and dox-off double-transgenic SCltTAxTRE-BCR-ABL mice (MPD). n=5 mice per group.

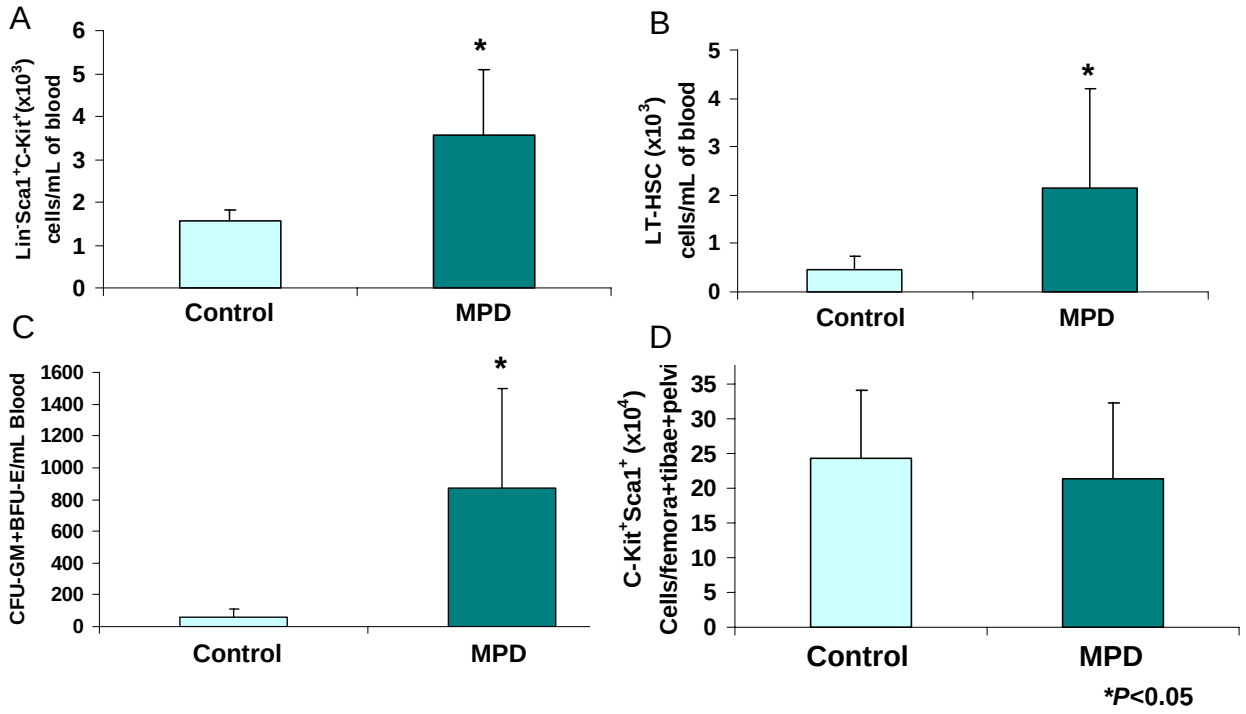


Figure 8. BCR-ABL transformation of stem cells and progenitors induces egression in vivo of leukemic cells from the BM niches to peripheral blood. A. Lin-/c-kit+/Sca-1+ circulating cells, B. Lin-c-kit+/Sca-1/CD34- (LT-HSC) in circulation; C. Myeloid/Erythroid hematopoietic progenitors in peripheral blood circulation; D. Lin-/c-kit+/Sca-1+ cells in BM.

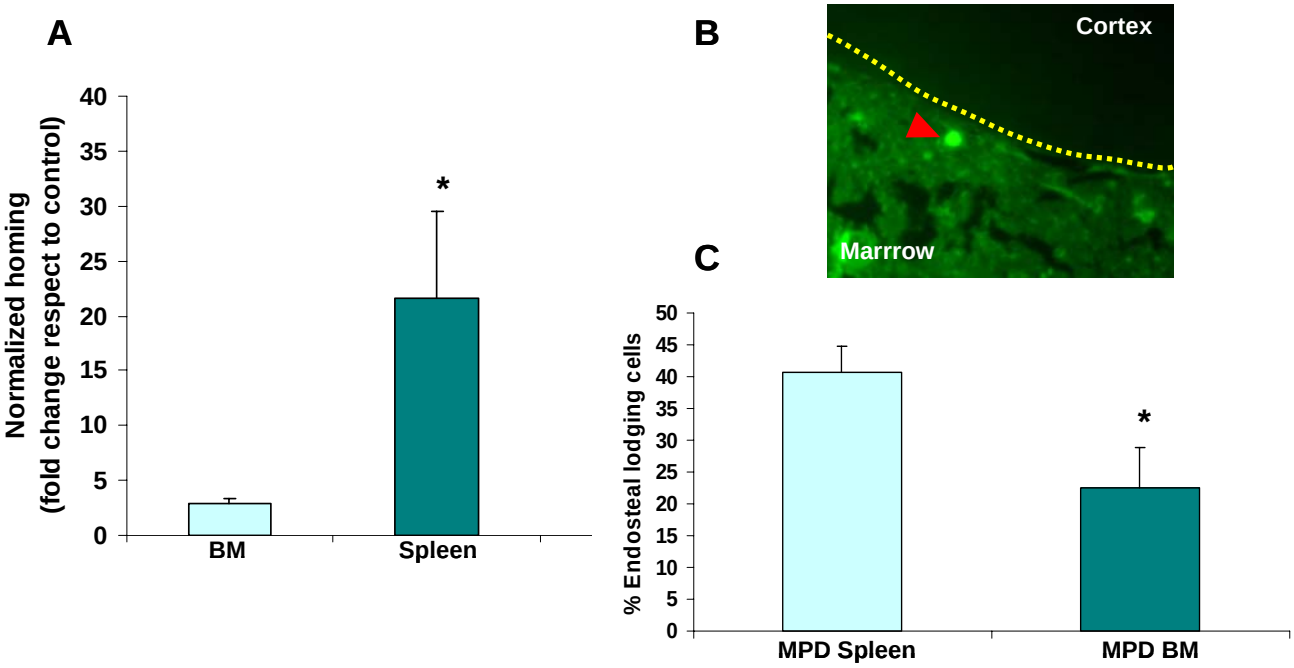


Figure 9. Homing efficiency and Endosteal localization. (A) Normalized fold homing (16h) of CFSE$^+$ lineage$^-$ SCL/p210 splenocytes to littermate recipient mice. Data represent mean±s.d. (n=3 mice per group; representative of 2 independent experiments). **(B, C)** Longitudinal section (4 µm) & frequency of endosteal lodged CFSE$^+$ cells transplanted either from MPD spleen or MPD BM. Data represent average of 2 independent experiments. *P<0.05.

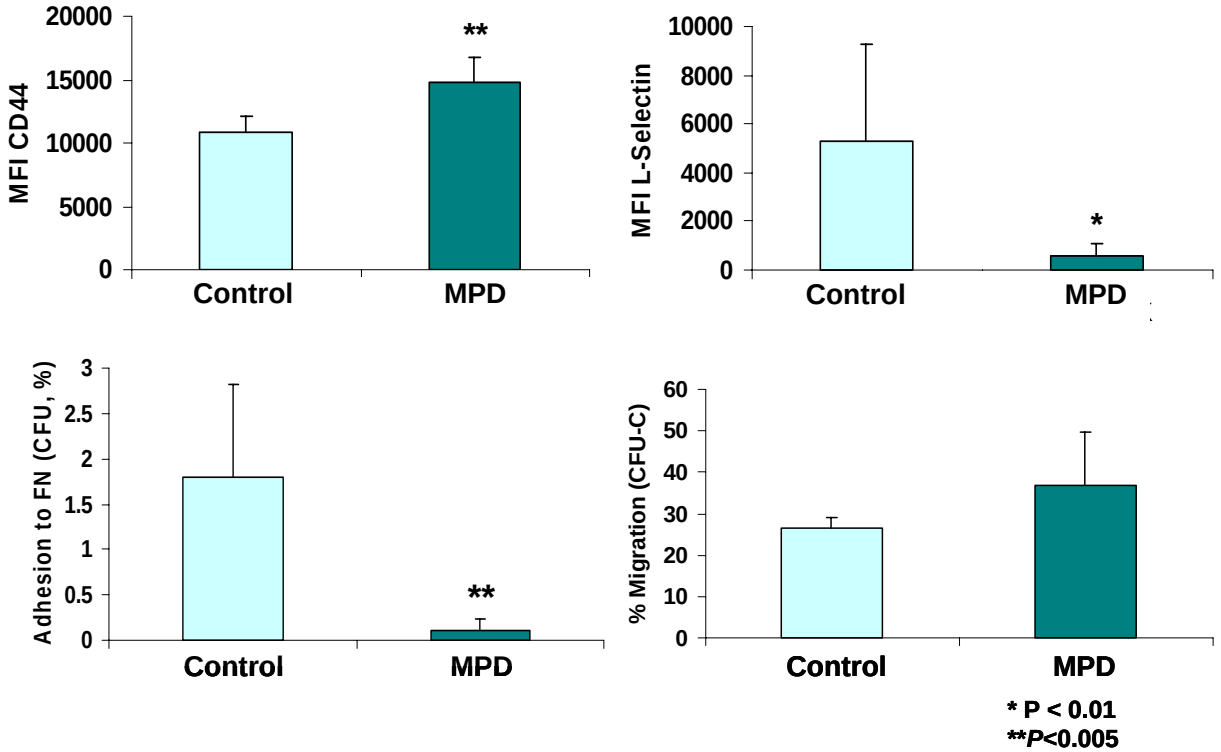


Figure 10. Adhesion molecule expression in leukemic stem cells and progenitors in mice expressing stem cell-driven p210-BCR-ABL is abnormal (A-B). Adhesion to Fibronectin (C) and migration towards CXCL12 (100 ng/mL, D) are also Impaired. N=2 independent experiments with a minimum of 3 mice per group).

Rac2 is a molecular target in p210-BCR-ABL-induced leukemic stem cell-initiated and maintained myeloproliferative disease.

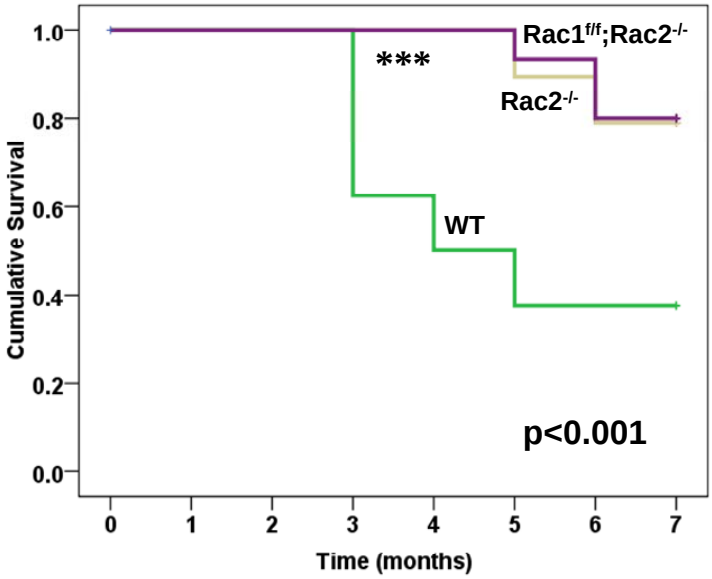


Figure 11. Cumulative survival of Rac2 and Rac1flox/flox;Rac2-deficient mice is increased in a stem cell-driven, homing independent murine model of CML. A minimum of 7 mice per group have been followed.

Confirming the results of our initiation murine model of disease initiated by transplantation of transformed hematopoietic progenitors, a murine model of stem cell-initiated disease which is homing independent, has shown that Rac2 is an in vivo molecular target for chronic phase CML **(Figure 11).** This impairment in leukemogenesis in vivo correlates with the observation that Rac2 deficient mice have a decreased leukemic HSC/P content in spleen **(Figure 12)**. Interestingly, Rac2 deficiency does not impair BM egression of HSC/P to peripheral blood in vivo **(Figure 13)** or adhesion or migration in vitro **(Figure 13)**, suggesting that others rather than migration are the transformation functions

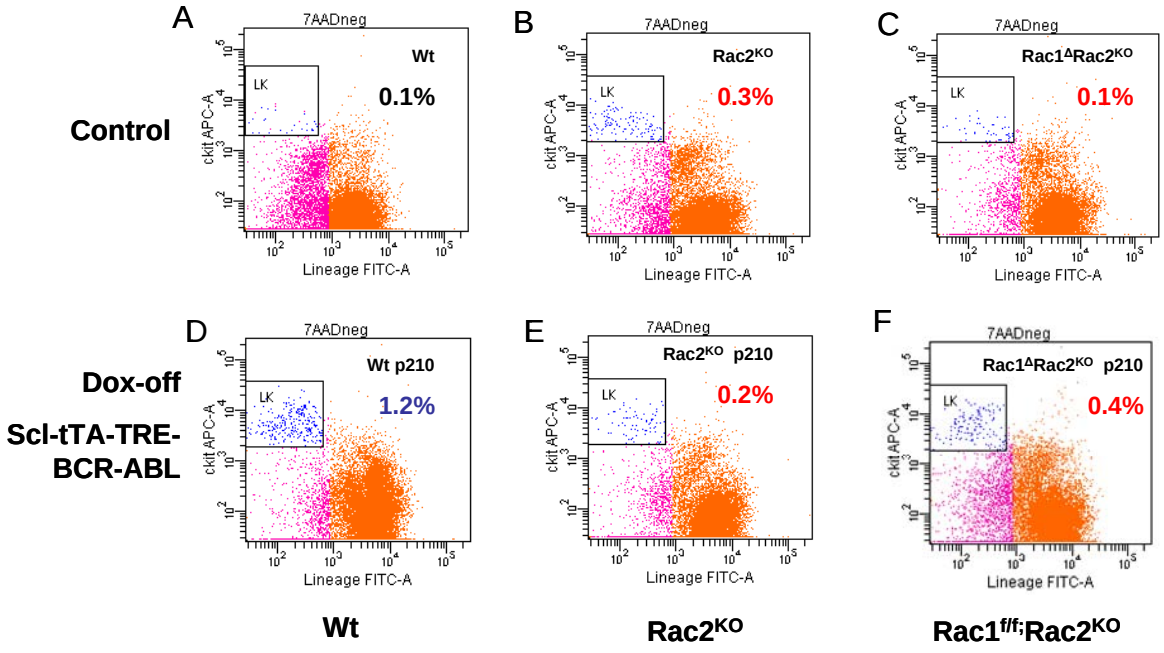


Figure 12. Flow cytometry example of a group of control (WT) mice (A-C) and CML mice (D-F) in presence (A,D) and absence of Rac expression (B,C,E,F). An increase of leukemic HSC/P (Lin-/c-kit+ cells) in CML mice is reduced in Rac2-deficient mice.

depending on Rac2.

We are now studying the other possible mechanisms responsible for Rac2-dependent leukemogenesis, focusing on analysis of survival of leukemic stem cells and progenitors in vitro and in vivo and on the downstream effectors that may be responsible of specific leukemogenic signals dependent upon Rac2 activation downstream BCR-ABL.

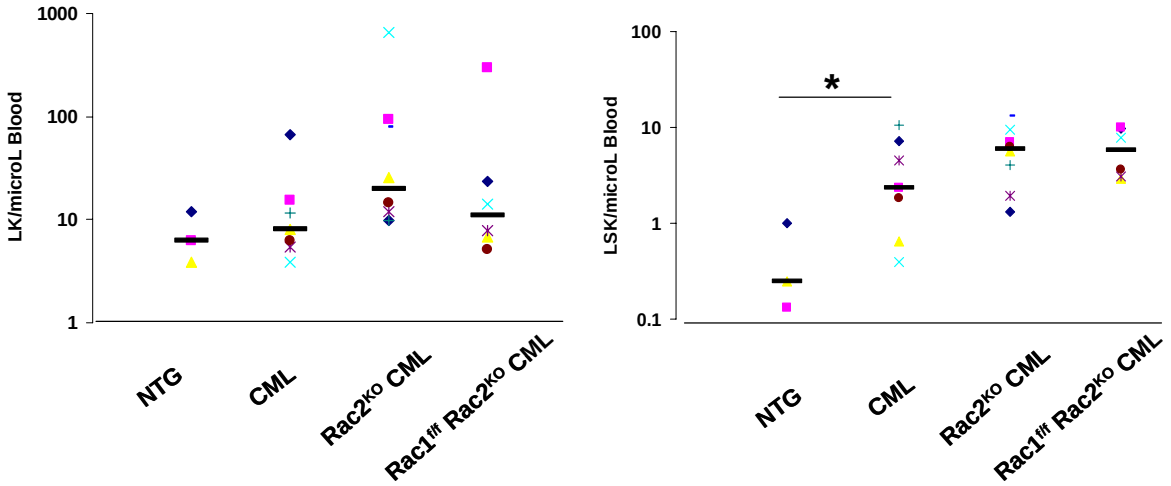
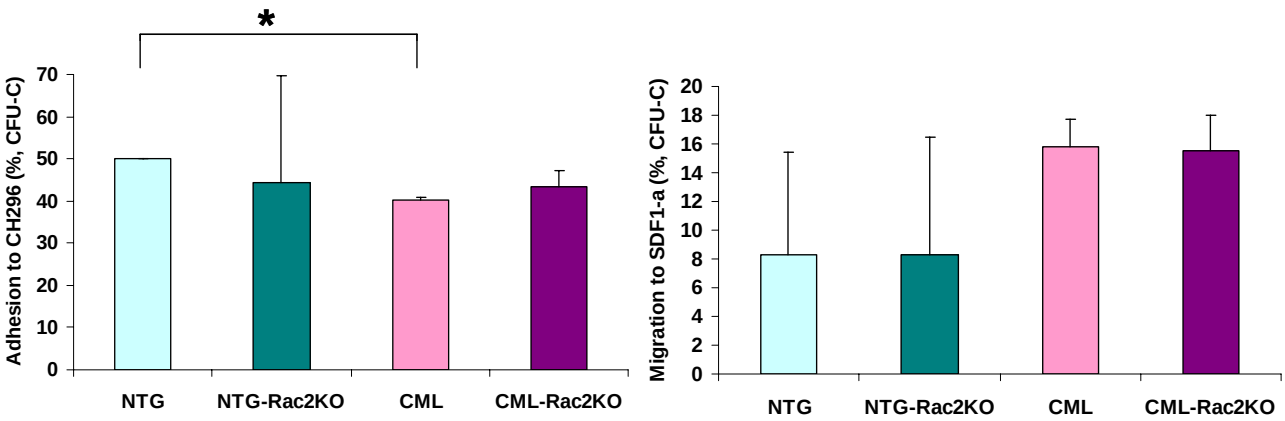


Figure 13. Rac2 deficiency does not modify the egression pattern to peripheral blood observe in CML (ScltTA x TRE-BCR-ABL, dox-off) mice, * p < 0.05.



* p<0.05 **n=3**

Figure 14. Adhesion to Fibronectin and migration of CML (Scl-tTAxTRE-BCR-ABL, dox-off) progenitors is not significantly affected by Rac2 deficiency.

Key research accomplishments

1. Rac3 isoform is not involved in CML initiation.

2. A murine model of leukemia initiation and maintenance, independent of homing of stem cells, shows has been validated showing downstream signaling, including CrkL, p38, Jnk and STAT3/5.

3. Gene targeting of *Rac2* significantly delays or abrogates disease initiation and/or maintenance in a homing-independent inducible murine model of CML.

Reportable outcomes

Manuscripts:

1. Williams DA, Zheng Y, Cancelas JA. Rho GTPases and regulation of hematopoietic stem cell localization. Methods in Enzymology 2008;439:365-93.
2. Thomas EK, Cancelas JA (co-first author), Zheng Yi, Williams DA. Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis. Leukemia 2008;439:365-93.
3. Müller LUW, Schore RJ, Zheng Y, EK Thomas, Kim M-O, Cancelas JA, Williams DA. Rac guanosine trisphosphatases represent a potential target in AML. Leukemia 2008 Sep;22(9):1803-6.

Abstracts

1. Sengupta A, **Cancelas** JA. Leukemic stem cells and progenitors demonstrate impaired interaction with the hematopoietic microenvironment in vivo in an inducible murine model of chronic myelogenous leukemia. Oral presentation (AS), Amer. Soc. Hematol. Annual Meeting, San Francisco, Dec 6-9, 2008. **Blood** 2008;112(11):78(191).
2. Sanchez-Aguilera A, Sengupta A, Mastin JP, Chang KH, Williams DA, **Cancelas** JA Rac2 GTPase activation is necessary for development of p190-BCR-ABL-induced B-cell acute lymphoblastic leukemia. Poster presentation (III-872), Amer. Soc. Hematol. Annual Meeting, San Francisco, Dec 6-9, 2008. **Blood** 2008;112(11):1297(3790).
3. Sanchez-Aguilera A, Sengupta A, Williams DA, **Cancelas** JA. Rac GTPase activation mediates BCR-ABL-induced B-cell acute lymphoblastic leukemia. Oral presentation at the 37th Annual Scientific Meeting of the ISEH. **Exp Hematol** 2008 Jul;36(7, Suppl 1):S3(6).
4. Sengupta A, **Cancelas** JA. Leukemic progenitor retention in the bone marrow niche is impaired in an inducible murine model of chronic myelogenous leukemia (CML). Presentation at the 37th Annual Scientific Meeting of the ISEH. **Exp Hematol** 2008 Jul;36(7, Suppl 1):S18.

Conclusion

We here have validated a murine model of inducible, homing-independent, chronic myelogenous leukemia. Rac2 appears to be confirmed as a molecular target for CML initiation and maintenance. Rac2 activity may not interfere with the migratory/adhesive transformation phenotype of BCR-ABL expressing stem cells but modifying other aspects of stem cell transformation like survival or proliferation. Rac3 deficiency does not impair leukemia initiation.

"So what?"

These data along with the previous data presented in the 2008 annual report, indicate that targeting Rac GTPases (specifically, Rac2) represents a novel therapeutic approach in the therapy of chronic phase CML, which probably allow the management of tyrosine-kinase resistant, chronic-phase CML and blastic-phase CML patients.

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effect—compared to those with grade IV, which is life threatening. However, results of subgroup analyses need to be interpreted with caution as subgroup comparisons across studies are entirely observational and thus have the same limitations as any observational study.

The analysis of chronic GvHD was hampered as the analysis in individual studies was based on different populations; to analyse chronic GvHD, the statistical guidelines for EBMT recommend to restrict the population to those patients who have survived at least until day 100 post transplant as only those are considered to be at risk. However, only two studies analysed their data following this recommendation.

The pooled results for overall survival and disease-free survival show that the advantage of decreased occurrences of acute GvHD grades I–IV and II–IV does not result in an advantage concerning the survival of patients receiving corticosteroids for preventing GvHD. This might be explained by the fact that a sole reduction of low grades of acute GvHD might not increase the survival rate as (a) especially higher grades have a negative impact on survival as explained above and (b) the beneficial graft-vs-leukaemia effect might be eliminated. The latter argument should be reflected in particular in the relapse rates, as less graft-vs-leukaemia effect is supposed to enhance the relapse rates. The conclusion, which can be drawn from the analysis of disease-free survival is, however, limited: the individual studies used various terms for this outcome (event-free survival, leukemia-free survival, and relapse-free survival) without stating the definitions used.

The occurrence of infectious complications is of special interest as (a) infections still remain an important cause of morbidity and mortality after allogeneic HSCT and (b) corticosteroids are non-specific immunosuppressive agents setting the stage more than others for infectious complications. Owing to the heterogeneity of the reported data and different outcome definitions, we could not perform a meta-analysis and thus, a final judgment about the influence of corticosteroids on the infection rate cannot be given. In addition, an interpretation is complicated by the fact that corticosteroids are used as first-line treatment for acute and chronic GvHD, which might contribute to high rates of infections—even if no corticosteroids are incorporated into the prophylaxis regimen.

In conclusion, there is strong evidence that the administration of corticosteroids to the prophylaxis regimen of GvHD reduces the occurrences of acute GvHD grade I–IV and II-IV. However, there is no evidence for a survival benefit. For the occurrence of infectious complications (under the concomitant use of antibacterial, antiviral and antifungal prophylactic medication), no conclusion can be drawn due to the heterogeneity of the reported data. The subgroup analyses indicated that a 'late' administration of the steroid administration, that is, not overlapping with MTX, might have a positive influence especially on acute GvHD. Further randomized controlled trials are needed to evaluate the impact of the timing of the steroid administration and to estimate the influence of corticosteroids on chronic GvHD analysed within a homogenously defined population and on the infection rate within a homogenously treated population.

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Rac guanosine triphosphatases represent a potential target in AML

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Acute myeloid leukemia (AML) is intrinsically prone to resistance to conventional chemotherapeutic agents. Here, we explore the Rac family of small guanosine triphosphatases (GTPases) as novel biologic targets for AML treatment. The Rac subfamily of Rho GTPases plays an essential role in regulating normal hematopoiesis.[1] Rac proteins cycle between active GTP-bound and inactive guanine diphosphate (GDP)-bound states. Of the three known Rac family members, Rac1 and Rac3 are expressed ubiquitously, whereas Rac2 is restricted to the hematopoietic system. In hematopoietic cells, Rac proteins integrate signals from growth factor, chemokine, and adhesion

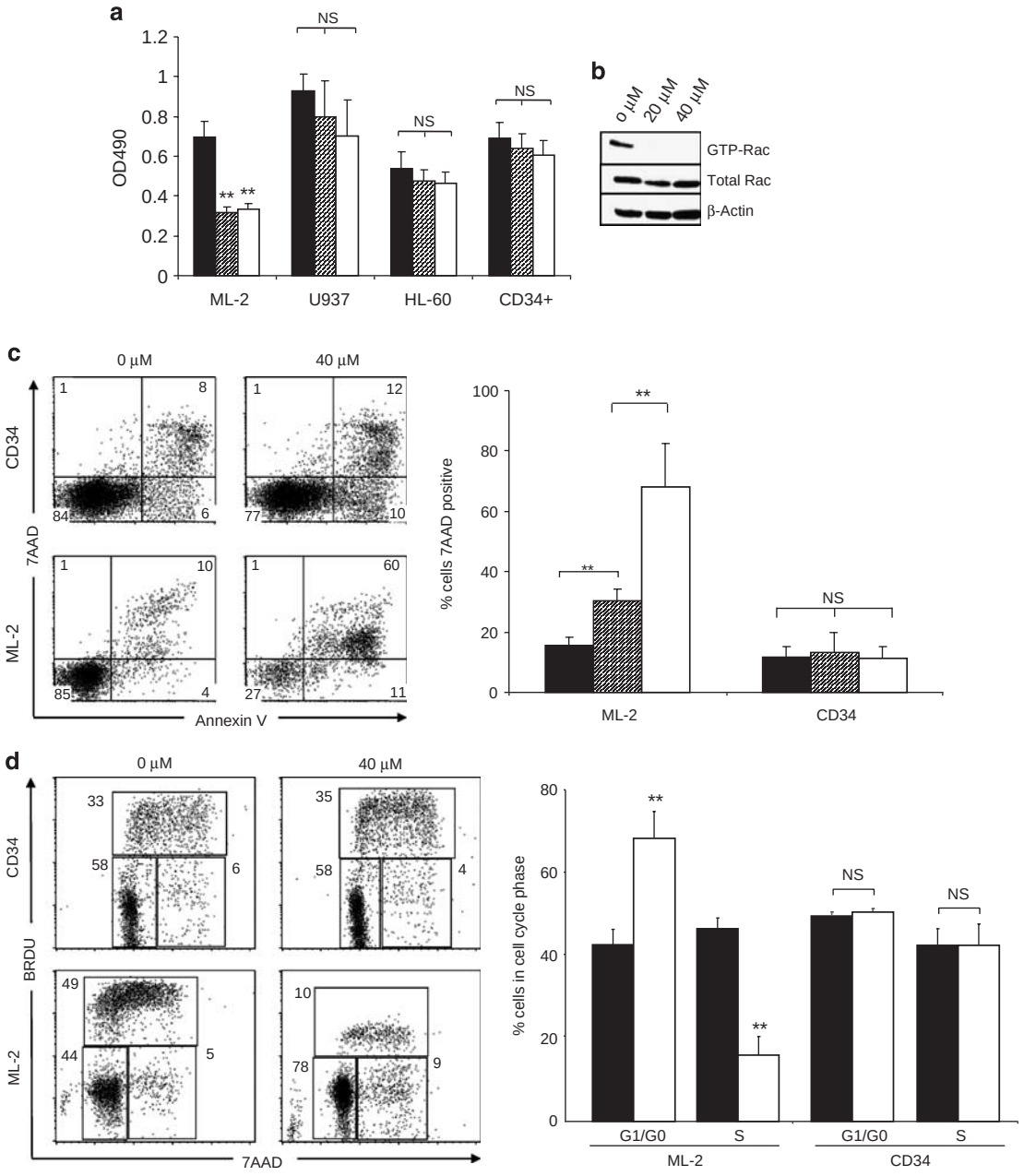

Figure 1 The Rac-specific inhibitor NSC23766 significantly impacts proliferation, survival and cell-cycle progression of human AML cell lines. (**a**) Proliferation of a panel of AML cell lines was analyzed by (3-(4,5)-dimethylthiazol-2-yl)-5-(3-carboxymethoxydenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay 72 h after exposure to increasing doses of NSC23766 ($n=3$, 24 wells per condition). Black bars indicate 0 μM, hatched bars indicate 20 μM and white bars indicate 40 μM NSC23766. (**b**) NSC23766 inhibits Rac activation in ML-2 cells. ML-2 cells were cultured in the presence of increasing doses of NSC23766. Lysates were analyzed for active GTP-Rac. As controls, total lysates were analyzed for Rac and actin expression. (**c**) Apoptosis of ML-2 and CD34+ cells was analyzed 72 h after exposure to NSC23766. Left panel depicts a representative dot blot analysis. Numbers indicate percentage of cells per quadrant. Graph depicts percentage of 7AAD-positive cells after exposure to 0 μM (black bars), 20 μM (hatched bars) or 40 μM (white bars) NSC23766 ($n=5$). (**d**) Cell-cycle analysis of ML-2 and CD34+ cells was performed 48 h after exposure to NSC23766. Left panel depicts representative dot blot after exposure to 0 or 40 μM NSC23766. Graph depicts the percentage of cells in G1/G0 or S phase of cell cycle 48 h after exposure to 0 μM (black bars) or 40 μM (white bars) NSC23766, respectively ($n=5$). $^{**}P<0.01$, NS, not significant. AML, acute myeloid leukemia.

receptors to mediate a variety of cellular responses, including cell growth and survival, gene transcription, adhesion, motility and formation of the actin cytoskeleton.[1] We recently identified the Rac GTPases as molecular targets in BCR-ABL-induced myeloproliferative disease.[2,3] However, the role of Rac in AML has thus far not been clearly defined. Elevated levels of GTP-bound Rac have been described in CD34+ cells isolated from patients with AML. In these samples, Rac signaling was identified as a critical mediator of stem/progenitor cell and stroma interaction.[4] Recently, Wei *et al.*[5] observed a critical role of Rac signaling in a disease model of human CD34+ cells transduced with the mixed lineage leukemia *(MLL)-AF9* fusion oncogene.[5] Interestingly, whereas *MLL-AF9* transduced cells were sensitive to Rac inhibition, cells transduced with the *AML-ETO* (ETO (eight twenty one)) oncogene did not depend on Rac signaling for survival and proliferation. These discrepancies

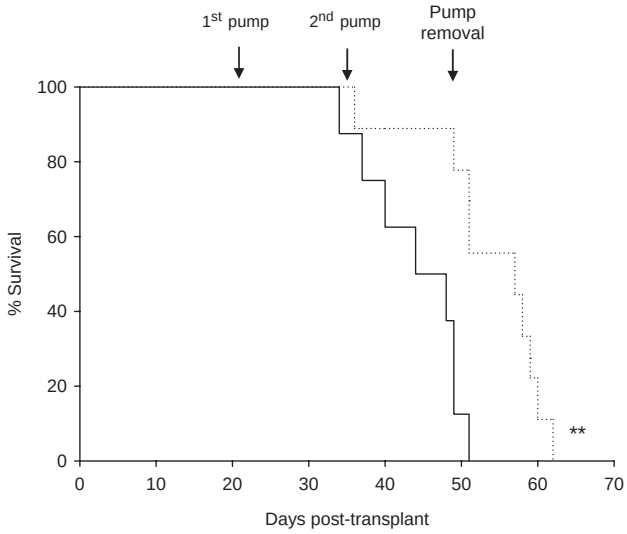

Figure 2 NSC23766 significantly delays the development of leukemia in a murine *in vivo* model. 2×10^7 ML-2 cells were transplanted into sublethally irradiated NOD/SCID mice. Arrows indicate placement/removal of Alzet osmotic pumps containing PBS (solid line, $n=8$) or NSC23766 (dotted line, $n=9$). $^{**}P<0.01$ (log-rank test).PBS, phosphate-buffered saline.

prompted us to further investigate the role of Rac signaling in a panel of human AML cell lines, including the MLL gene-rearranged ML-2 cell line and cell lines not harboring MLL rearrangements such as the histiocytic lymphoma U937 and the acute promyelocytic HL-60 cell line. We demonstrate the presence of GTP-Rac in all cell lines through p21-activated kinase-binding domain pull down and immunoblot (data not shown). Compared to purified normal human CD34+ cells, ML-2 cells, which contain a MLL-AF6 translocation,[6] showed the most profound inhibition of cell proliferation upon pharmacologic inhibition of Rac using the small molecule Rac inhibitor NSC23766[7] (Figure 1a). To determine whether a correlation exists between Rac activation and the observed decrease in proliferation of ML-2 cells, we analyzed the effect of NSC23766 on GTP-Rac through the p21-activated kinase-binding domain pull-down assay and observed abrogation of Rac activation with drug treatment (Figure 1b). We next wanted to determine whether NSC23766 treatment would impact apoptosis (Figure 1c) and/or cell-cycle progression of ML-2 cells (Figure 1d). ML-2 cells displayed an increase of early and late apoptosis as measured by Annexin V/7AAD 72 h after drug exposure (Figure 1c). In addition, Rac inhibition led to increased cell-cycle arrest in G0/G1 (Figure 1d). Importantly, these effects were specific to ML-2 cells, as normal CD34+ cells were not significantly affected by NSC23766 exposure. Analogous effects were observed in the MLL-AF9-containing THP-1 cell line (Supplementary Figure 1). In contrast to these MLL gene rearranged cell lines, no significant effect of NSC23677 on cell cycle or apoptosis was observed in U937 cells and only marginal effects were observed in HL-60 cells (data not shown). To further analyze the potential therapeutic efficacy of NSC23766 in a murine xenograft model, 2×10^7 ML-2 cells were transplanted into irradiated (350 Gy) non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Alzet osmotic pumps containing NSC23766 (two pumps, 75 mM NSC23766 per pump) or phosphate-buffered saline (PBS) were implanted on day 21 post-transplant. The pumps were exchanged for new pumps on day 35 and removed on day 49 post-transplant. Animals were monitored for survival and bone marrow chimerism of ML-2 cells (human CD45+) was assessed by flow cytometry postmortem. Animals with less than 15% human CD45+ chimerism were censored from the study (three animals in the NSC23766- and four animals in the PBS cohort). We noted a significant difference in the survival of PBS vs NSC23677-treated animals (Figure 2). Death due to disease progression in the treatment group occurred predominantly following pump removal.

In summary, we demonstrate the impact of Rac inhibition on a panel of human AML cell lines. Whereas the drug target GTP-Rac was present in all cell lines (not shown), the MLL gene-rearranged cell line ML-2 displayed the most profound dependence on Rac signaling. This finding is consistent with recently published findings in human CD34+ cells transduced with the *MLL-AF9* oncogene and corroborated by our findings in the MLL-AF9-positive THP-1 cell line.[5] The effect of NSC23766 may point to a potential specific vulnerability of MLL rearranged leukemia to Rac inhibition. The mechanism by which the other cell lines bypass this pathway will require further studies. Taken together, our findings highlight the Rac GTPases as potential molecular targets for a subgroup of AML. Development of more potent inhibitors of Rac GTPases is warranted to enable clinical use of this approach.

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Multiple sub-microscopic genomic lesions are a universal feature of chronic myeloid leukaemia at diagnosis

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Chronic myeloid leukaemia (CML) is a clonal stem cell disorder, characterized at the cytogenetic level by the presence of a balanced chromosomal rearrangement, the t(9;22) or Philadelphia chromosome (Ph) translocation and at the molecular level by the presence of the *BCR-ABL* fusion gene.[1] Several lines of evidence point to deregulated expression of the *BCR-ABL* tyrosine kinase as the initial genomic lesion in CML.[1]

Despite the presence of a consistent genetic abnormality, however, CML patients display considerable clinical heterogeneity, the basis of which is poorly understood. This heterogeneity was well characterized by Sokal *et al.*[2] and is reflected 24 years later by the varying responses to treatment in chronic phase patients treated with a tyrosine kinase inhibitor.[3] We therefore used a novel ultra-high-resolution genomic screening assay to search for additional acquired genomic abnormalities that might explain this clinical heterogeneity and help to assess prognosis for individual patients.

DNA was extracted from the polymorphonuclear cells in bone marrow samples from 10 previously untreated chronic phase patients. These patients subsequently received imatinib and achieved complete cytogenetic responses, at which point further polymorphonuclear-derived DNA was prepared. Comparative genomic hybridization (CGH) was performed with a 2.1 million oligonucleotide array (NimbleGen, Milton Keynes, UK; 'HD2' 070713_HG18_WG_CGH_HX1 design). The probes on this array were selected to achieve a uniform distribution throughout the genome, with approximately one probe every 1200 bp. Each DNA sample from diagnosis was competitively hybridized against the same patient's remission sample, which avoided detection of constitutional polymorphic copy number variants and limited results to acquired leukemia-related changes. Scanned array images were imported into NimbleScan (NimbleGen) to identify copy number aberrations (CNAs) from HD2 image and intensity data. Nexus 3 software (BioDiscovery Inc., El Segundo, CA, USA) was used to visualize the normalized segmented data. For representative CNAs the CGH result was confirmed by fluorescence *in situ* hybridization or quantitative real-time PCR.

All 10 CML patient samples harboured detectable genomic imbalances with an average of 53 CNAs per patient (range: 4–166). Of the 530 CNAs detected 381 (72%) were amplifications and 149 (28%) were deletions. Two hundred and fifty two CNAs (48%) involved at least one known gene. Many of the CNAs that involved single genes contained the complete gene with only small quantities of adjacent non-coding DNA. The average size of CNAs was 103 kb (range 9 kb–2 Mb). Seventy different genomic regions were aberrant in two or more patients in the cohort. Of these recurrent CNAs, amplifications of the *DUSP1* and *PBEF1* genes were most frequently detected; they were present in four and eight patients respectively. Other genes amplified or deleted in more than one patient included *DUSP22*, *MAPK8IP1*, *MAP3K11*, *SUPT4H1*, *PTPRC*, *GRK6* and several members of the histone gene family.

The HD2 platform provides a sensitivity that is at least an order of magnitude greater than that of those employed in previous studies. Brazma *et al.*[4] used a bacterial artificial chromosome array with a much lower resolution (1 Mb) and discovered 14 common cryptic abnormalities in CML blast crisis samples that were rare in chronic phase. In a comparison between Ph-positive acute lymphoblastic leukaemia and CML, Mullighan *et al.*[5] used a higher-resolution 250 k SNP array and observed up to 8 CNAs per patient (range 0–8) in a cohort of 23 chronic phase CML samples, but no recurrent aberrations were detected and the authors did not specify whether samples had been taken at diagnosis or later in chronic phase. Exclusive analysis of presentation samples in our study demonstrates that many CNAs are early events in CML. Furthermore, the use of the patients' remission DNA as reference material rather than pooled normal DNA confirms all observed imbalances as disease-related.

The findings of our ultra-high-resolution screening have numerous implications. The presence of multiple genomic lesions at diagnosis supports the notion of an increased level of genomic instability in CML cells and raises the possibility that one or more aberrations in addition to *BCR-ABL* may dictate the CML phenotype. The considerable range in the number of CNAs present at diagnosis would be consistent with a differing level of genomic instability between individual patients. Assessment of the correlation between number of CNAs detected at diagnosis and survival in an unselected group of patients might show that high-resolution genome profiling was a good method for predicting clinical outcome in CML.

The observation that many CNAs are demarcated by the extremities of individual genes, suggests that these genes might be specific targets. Furthermore, the presence of same genetic imbalances in more than one patient in the cohort suggests a role for these genes in the pathogenesis or progression of CML. The most commonly involved gene, *PBEF1*, has a number of functions including roles in neutrophil proliferation[6] and in the NAD anti-apoptotic pathway.[7] Expression of *PBEF1* is modulated by *JUN-B*,[8] which is commonly downregulated in CML cells.[1] It is therefore plausible that *PBEF1* could be involved in the pathogenesis of CML. *DUSP1*, overrepresented in 4 of our 10 patients, might be a cooperating gene as it regulates mitogen-

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SPOTLIGHT

REVIEW

Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis

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Chronic myelogenous leukemia (CML) is a malignant disease characterized by expression of p210-BCR-ABL, the product of the Philadelphia chromosome. Survival of CML patients has been significantly improved with the introduction of tyrosine kinase inhibitors that induce long-term hematologic remissions. However, mounting evidence indicates that the use of a single tyrosine kinase inhibitor does not cure this disease due to the persistence of p210-BCR-ABL at the molecular level or the acquired resistance in the stem cell compartment to individual inhibitors. We have recently shown in a murine model that deficiency of the Rho GTPases Rac1 and Rac2 significantly reduces p210-BCR-ABL-mediated proliferation *in vitro* and myeloproliferative disease *in vivo*, suggesting Rac as a potential therapeutic target in p210-BCR-ABL-induced disease. This target has been further validated using a first-generation Rac-specific small molecule inhibitor. In this review we describe the role of Rac GTPases in p210-BCR-ABL-induced leukemogenesis and explore the possibility of combinatorial therapies that include tyrosine kinase inhibitor(s) and Rac GTPase inhibitors in the treatment of CML.
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Introduction

The p210-BCR-ABL fusion protein that is generated from a reciprocal translocation between the breakpoint-cluster region (BCR) gene on Chromosome 22 and the Abelson leukemia (ABL) gene on Chromosome 9 is necessary and sufficient for the development of chronic myelogenous leukemia (CML).[1,2] Although allogeneic stem cell transplantation is a curative therapy for the treatment of CML, most patients lack suitable donors or are not eligible for transplant due to advanced age.[3–6] The development of imatinib mesylate, a tyrosine kinase inhibitor that has been shown to induce complete hematologic and cytogenetic responses in many patients, has provided an effective means of treatment of CML and has rejuvenated the field of rationalized drug design.[7] Imatinib targets the abnormal kinase activity of CML blasts and induces apoptosis of p210-BCR-ABL$^+$ cells.[8,9] However, in a proportion of patients the persistence of p210-BCR-ABL$^+$ cells or the development of p210-BCR-ABL kinase mutants that confer resistance to imatinib have been demonstrated.[10,11] While a second generation of tyrosine kinase inhibitors including nilotinib and dasatinib are effective at inhibiting the activities of most imatinib-resistant p210-BCR-ABL mutants, the use of sequential Abl kinase inhibitor therapy has been shown to select for compound mutations that confer resistance to both drugs and increase p210-BCR-ABL oncogenicity,[12] suggesting that other signaling components downstream of p210-BCR-ABL should be considered as potential therapeutic targets.

The Rac subfamily of Rho GTPases comprising the highly related mammalian proteins Rac1, Rac2 and Rac3 has previously been implicated in p210-BCR-ABL-mediated transformation using cell lines and in acute myelogenous leukemia (AML) cell migration dependent on vascular endothelial growth factor paracrine stimulation.[13] Rac1, in particular, has been identified as an important downstream component of BCR-ABL signaling, suggesting Rac GTPases as possible molecular targets for interrupting abnormal signaling in CML blasts.[14–19] Our findings that the combinatorial loss of Rac1 and Rac2 significantly attenuates p210-BCR-ABL-induced proliferation *in vitro* and myeloproliferative disease (MPD) *in vivo* provide additional genetic evidence that the Rac GTPases may be attractive therapeutic targets in p210-BCR-ABL-mediated MPD.[20] These genetic data were further substantiated experimentally by use of NSC23766, a first-generation small molecule inhibitor that specifically blocks activation of the Rac GTPases. While our data confirm that Rac GTPases are candidate therapeutic targets in p210-BCR-ABL-mediated disease, a number of questions remain regarding the role of Rac and other Rho GTPases in p210-BCR-ABL-induced leukemogenesis.

Relationship between Rac GTPases and p210-BCR-ABL in CML

The Rac subfamily of Rho GTPases has been implicated in a variety of different cellular functions, including adhesion, migration, actin assembly, transcription activation, cell cycle progression and cell survival (reviewed in Blanchard[21]). Similar to other Ras-related GTPases, Rac GTPases cycle between inactive, GDP-bound and active, GTP-bound conformations to transduce signals to effector proteins that mediate a multitude of cellular responses. Three structurally related proteins, Rac1, Rac2 and Rac3, have been identified. While Rac1 and Rac3 are ubiquitously expressed, expression of Rac2 is restricted to hematopoietic tissues. Thus, hematopoietic cells are unique in expressing all three Rac proteins. Our laboratory has previously shown that Rac1 and Rac2 are essential for the regulation of multiple hematopoietic stem cell functions with unique as well as overlapping roles, including adhesion, migration, proliferation and apoptosis.[22] In effecting these responses, Rac GTPases

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have been shown to activate signaling molecules that coincide with known downstream targets of p210-BCR-ABL,[18,19] such as the Ras/MAP kinases (ERK, p38 and JNK), phosphatidylinositol-3-kinase (PI3K)/Akt, Bcl-X_L and focal adhesion kinase (FAK).

These earlier observations highlight a possible relationship between Rac GTPases and p210-BCR-ABL, although the specific role(s) of the individual Rac subfamily members in the development of disease *in vivo* have not previously been defined. Skorski *et al*.[16] showed that activation of Rac GTPases is enhanced in 32Dcl3 myeloid precursor cells ectopically expressing p210-BCR-ABL. Additionally, survival of mice injected with 32Dcl3 cells co-expressing p210-BCR-ABL and a dominant-negative N17Rac mutant was markedly extended, compared to mice transplanted with 32Dcl3 cells expressing p210-BCR-ABL alone.[16] Harnois *et al*.[17] showed that the p210-BCR-ABL fusion protein forms a stable complex with Rac1, Rac2, and other RhoGTPases including RhoA and Cdc42 and may directly activate these Rho GTPases through the Dbl homology domain of Bcr. Conversely, these Rho GTPases may be activated by p210-BCR-ABL through the recruitment of Vav1,[17] a hematopoietic-specific guanine nucleotide exchange factor (GEF) that is crucial for Rac activation in lymphoid[23] and other hematopoietic cells. Sini *et al*.[14] have described activation of Rac by Abl-induced tyrosine phosphorylation of Sos-1, which could be inhibited by genetic or pharmacological inhibition of Abl. Additionally, p210-BCR-ABL has previously been shown to display a Rac-dependent induction in transformation-associated changes in cytoskeletal functions such as actin assembly, migration and adhesion, all known functions of Rho GTPases, particularly Rac.[18,19] Finally, Diaz-Blanco *et al*.[24] have recently shown that Rac1 and Rac2 were significantly upregulated in CD34+ human chronic phase CML bone marrow (BM) cells. These and other data imply that p210-BCR-ABL interacts directly and/or indirectly with Rac, Rho and Cdc42 to activate these GTPases in cell lines.

In support of this postulated involvement of Rac GTPases in p210-BCR-ABL-mediated disease, we have demonstrated hyperactivation of Rac1 and Rac2 and, to a lesser extent, Rac3 in hematopoietic stem cells and progenitors (HSC/P) isolated from chronic phase CML patients. These data confirm that Rac GTPases are abnormally activated in chronic phase disease. Experimentally, Rac GTPases were also shown to be hyperactivated in primary murine BM cells expressing p210-BCR-ABL after retrovirus-mediated gene transfer.[20] To determine the importance of the individual Rac GTPases in the development of p210-BCR-ABL-mediated CML, we employed an *in vivo* retroviral murine model of hematopoietic stem cell transformation combined with the use of BM cells from gene-targeted mice to effect deletion of Rac1 alone, Rac2 alone and Rac1 in combination with Rac2. As originally described,[1] this model has demonstrated that expression of p210-BCR-ABL in murine HSC/P can induce an MPD, including the development of leukocytosis, splenomegaly, extramedullary hematopoiesis in the liver and pulmonary hemorrhage due to extensive granulocyte infiltration in the lung. In our reported studies, while the median survival of p210-BCR-ABL-expressing wild-type (WT) and Rac1-deficient mice was 23 and 22 days, respectively, the median survival of p210-BCR-ABL-expressing Rac2-deficient mice was significantly increased to 43 days, and the median survival of p210-BCR-ABL-expressing Rac1/Rac2-deficient mice was even more strikingly increased to 92 days. This result suggests that individual Rac GTPases play unique roles in p210-BCR-ABL-mediated leukemogenesis, as has been described for normal HSC/P functions.[22,25]

Using this genetic approach, we also monitored the disease phenotype of the p210-BCR-ABL-expressing WT and Rac-deficient animals. Expression of p210-BCR-ABL in WT, Rac1- and Rac2-deficient HSC/P led to the development of oligoclonal myeloid-lineage leukemias. Expression of p210-BCR-ABL in Rac1/Rac2-deficient HSC/P led to altered disease phenotype, with mice showing oligoclonal leukemias of myeloid, lymphoid or bi-lineage immunophenotypes, suggesting that Rac1 and Rac2 are critical for transformation and MPD development *in vivo*. The mechanism of these differences in disease phenotypes is still being investigated but could be related to alterations in downstream signaling pathways in the absence of Rac1 and Rac2 and/or compensatory alterations in the activity of Rac3.

Loss of Rac GTPases alters the signaling cascades activated by p210-BCR-ABL

The results presented above suggest that Rac1 and Rac2 play an important role in the development of p210-BCR-ABL-mediated MPD, but in the absence of these GTPases, expression of p210-BCR-ABL can lead to the eventual progression of phenotypically altered disease via unspecified downstream signaling components. What is the mechanism by which p210-BCR-ABL mediates disease in the absence of Rac1 and Rac2? To answer this question, we first analyzed the status of Rac3 activation in splenocytes harvested from leukemic Rac1/Rac2-deficient animals. Rac3 is the third member of the Rac subfamily of Rho GTPases that was originally discovered by screening the p210-BCR-ABL-expressing erythroid blastic-phase CML cell line K562.[26] Rac3 activation has been demonstrated in p190-BCR-ABL-expressing malignant precursor B-lineage lymphoblasts[27] and is associated with the invasive phenotype of breast carcinomas,[28,29] suggesting that Rac3 hyperactivation could play a specific role in cancer development and invasiveness. We demonstrated that Rac3 was hyperactivated in p210-BCR-ABL-expressing leukemic animals in the absence of Rac1 and Rac2.[20] These data, along with the observed differences in survival mediated by Rac1- versus Rac2-deficient HSC, support the hypothesis that individual Rac GTPases play unique roles in the development of p210-BCR-ABL-mediated disease. Studies are underway to further explore the specific roles of each Rac GTPase in disease evolution and phenotype.

Mice that express p210-BCR-ABL with a point mutation in the ATP-binding site of ABL do not develop leukemia.[30] This indicates that ABL kinase activity is required for p210-BCR-ABL-induced transformation. However, the p210-BCR-ABL fusion protein is also composed of several structural domains that play distinct roles in cell signaling. Phosphorylation of Bcr at tyrosine 177 recruits Grb2/Gab2 and Sos, which results in Ras, ERK, JNK and p38 MAP kinase activation.[31,32] Phosphorylation of Bcr at tyrosine 177 also leads to the recruitment of SHP2 and PI3K/Akt.[33] Ras may be activated by two additional substrates of p210-BCR-ABL, the adapter molecules Shc and CrkL.[34,35] The actin-binding domain and the C-terminal domain of ABL, while not necessary for p210-BCR-ABL-mediated leukemogenesis experimentally, may contribute to the malignant behavior of p210-BCR-ABL leukemic blasts.[36] As mentioned previously, the role of a Dbl homology domain present in p210-BCR-ABL but not in a shorter form of BCR-ABL (p190-BCR-ABL) remains controversial. This domain may mediate Rac activation[17] that has been demonstrated to be necessary for full Ras-mediated transformation.[16] Finally, the Src homology domains SH2 and SH3 of p210-BCR-ABL bind to the Src family kinase member Hck and can phosphorylate signal transducer and activator of

transcription 5 (STAT5) independently of Janus kinase activation.[37] These known signaling functions of the fusion protein suggest that p210-BCR-ABL-mediated signals may converge on Rac through several pathways such as Ras/MAPK (ERK, p38 and JNK), PI3K/Akt, Bcl-x_L and FAK to alter proliferation and survival.

We analyzed activation of ERK, JNK, p38, Akt, STAT5 and CrkL in splenocytes harvested from p210-BCR-ABL-expressing WT, Rac1-deficient, Rac2-deficient and Rac1/Rac2-deficient animals. Increased baseline phosphorylation of each of these signaling components was apparent in cells derived from leukemic WT animals, as well as Rac1-deficient leukemic animals. However, activation of downstream pathways including ERK, JNK, p38 and Akt was attenuated in Rac2-deficient leukemic cells and almost completely abrogated in the Rac1/Rac2-deficient cells, correlating with the overall survival that was observed in animals from each of these genotypes. The decreased activation of downstream pathways was not due to decreased ABL tyrosine kinase activity, as autophosphorylation of p210-BCR-ABL was still noted in these cells.[20] STAT5 phosphorylation also was still detectable in leukemic cells regardless of the presence or absence of Rac1 and Rac2 GTPase activity. These data suggest that STAT5 may be the crucial signaling component for leukemia development in Rac1/Rac2-deficient HSC/P.

Surprisingly, activation of CrkL, which has been suggested to be an effector that binds directly to p210-BCR-ABL,[34] was decreased in Rac2-deficient and practically abrogated in Rac1/Rac2-deficient leukemias. CrkL activation has recently been reported to be dependent on a large multimeric protein complex that contains at least PI3K, docking protein 2 (DOK2), CrkL, Vav and Rac.[38,39] Thus, our data support a hypothesis that Rac participates in the activation of CrkL in the context of a multiprotein complex.

Putative role of STAT5 in the development of Rac3-mediated MPD

As mentioned previously, Rac3 was found to be hyperactivated in Rac1/Rac2-deficient leukemic animals, suggesting that this GTPase may be important in the eventual development of disease. These results imply that either the individual Rac GTPases play specific roles in the development of p210-BCR-ABL-mediated disease or the combinatorial loss of Rac expression modulates the disease phenotype. Interestingly, STAT5 activation was also apparent in Rac1/Rac2-deficient leukemic animals, suggesting that disease development also may be modulated by this protein.

STAT5 activation has been shown to be pivotal in myeloid differentiation[40] and multiple groups have demonstrated a critical role of STAT5 in the pathogenesis of CML,[41–45] but STAT proteins in leukemic transformation remain highly controversial. The physical interaction of p210-BCR-ABL and STAT5 was delineated by Nieborowska-Skorska *et al*.[46] using retroviral expression of BCR-ABL mutants in 32Dc13 cells. They showed that deletion of the SH2 domain accompanied by a point mutation in the SH3 domain of p210-BCR-ABL abolished STAT5 activation, as did deletion of both the SH2 and SH3 domains. Nieborowska-Skorska *et al*.[46] also demonstrated that cells expressing these STAT5 activation-deficient p210-BCR-ABL mutants were more apoptotic than cells expressing unmutated protein. Additionally, a constitutively active STAT5 mutant was able to rescue cells expressing STAT5-deficient BCR-ABL mutants from apoptosis while 32Dcl3 cells co-expressing a dominant-negative STAT5 mutant and BCR-ABL still underwent apoptosis, confirming the protective effect of STAT5.[46]

Sillaber *et al*.[47] demonstrated that inducible expression of a truncated STAT5 protein (ΔSTAT5) could dimerize with endogenous STAT5 and inhibit STAT5-induced gene transcription and growth in p210-BCR-ABL-expressing Ba/F3 hematopoietic cells, suggesting that STAT5 activation was responsible for most of the cell growth induced by p210-BCR-ABL. Expression of ΔSTAT5 resulted in inhibition of STAT5-induced transcription and a significant reduction in cell growth due to decreased cell viability and greater cell sensitivity to cytotoxic agents such as hydroxyurea and cytarabine.[47] Ba/F3 cells transfected with a vector expressing the Y177F mutant of BCR-ABL exhibited decreased tyrosine phosphorylation and activation of STAT1 and STAT5, compared with transfectants expressing wild-type BCR-ABL, suggesting that phosphorylation of Tyr-177 may be important for the activation of STAT signaling pathways by BCR-ABL. Tyrosine 177 of BCR-ABL has previously been shown as critical for binding to the adaptor protein GRB2, which mediates Ras/MAP kinase activation, suggesting that STAT5 activation may depend on Ras/Rac activation. Finally, STAT5 activation mediated by autocrine secretion of granulocyte-macrophage colony-stimulating factor has recently been shown to be responsible for the outgrowth of imatinib-resistant CML,[48] implying that STAT5 may be an alternative escape pathway by which leukemic cells circumvent tyrosine kinase inhibition.

Conversely, other studies suggest that STAT5 is not important in the pathogenesis of CML. Specifically, lethally irradiated mice transplanted with p210-BCR-ABL-expressing STAT5a/b N-terminal deletion mutant (Stat5a/b$^{\Delta N/\Delta N}$) cells developed disease as rapidly as mice injected with p210-BCR-ABL-expressing WT cells, suggesting that STAT5 is not essential for the development of p210-BCR-ABL-mediated disease.[49] Similar to our findings with Rac1-/Rac2-deficient BM, the majority of the p210-BCR-ABL-expressing WT mice developed myeloid lineage leukemias, while the p210-BCR-ABL-expressing STAT5a/b N-terminal deletion mutant mice had either myeloid, lymphoid or bilineage leukemias.[49] Interestingly, when the entire Stat5a/b gene locus was deleted, p185-BCR-ABL-expressing Stat5a/b$^{null/null}$ cells were resistant to transformation and did not induce lymphoid leukemia development in mice,[50] suggesting that STAT5 is critical for the development of p185-BCR-ABL-mediated disease. Whether p210-BCR-ABL-expression in the absence of Stat5 (that is, in Stat5a/b$^{null/null}$ cells) can modulate the development of MPD is unknown, but represents an intriguing question. Due to these discrepancies, the role of STAT5 in p210-BCR-ABL-mediated disease needs to be further characterized.

Compensatory hyperactivation of Rac3 in the absence of Rac1 and Rac2 may be responsible for the eventual disease development that is observed. The question now remains whether this Rac3-mediated disease is due to activation of the STAT5 signaling cascade.

Rac GTPases as targets for p210-BCR-ABL-mediated CML therapy

On the basis of these genetic data, we examined the effect of NSC23766 on p210-BCR-ABL-induced transformation. NSC23766 is a first-generation, Rac-specific small molecule inhibitor[51] that was developed based on the GEF-Rac1 GTPase complex and computer-assisted virtual screening. NSC23766 was found to fit into a shallow surface groove of Rac1 that has been shown to be critical for GEF specification. In published

studies, NSC23766 was shown to effectively inhibit Rac protein binding and activation by the Rac-specific GEFs TrioN or Tiam1 in a dose-dependent manner. In contrast, NSC23766 did not interfere with the binding or activation of Cdc42 or RhoA by their respective GEFs. In cells, NSC23766 effectively blocked serum- or platelet-derived growth factor-induced Rac1 activation and lamellipodia formation, but did not affect endogenous Cdc42 or RhoA activity. NSC23766 reduced growth stimulated by the Rac-GEFs Trio or Tiam1, but not proliferation stimulated by the promiscuous Rho/Cdc42 GEFs such as Dbl, Lbc, intersectin or a constitutively active Rac1 mutant. Importantly, NSC23766 suppressed Trio, Tiam1 or Ras-induced cell transformation and was shown to attenuate solid tumor cell line transformation and invasion. When human prostate cancer PC-3 cells were treated with NSC23766, Rac1 activity was downregulated, and proliferation, anchorage independent growth and invasion phenotypes that require endogenous Rac1 activity were inhibited.[51]

We have previously shown that retention of murine HSC/P is a Rac-dependent function.[22,25,52] On the basis of initial observations of NSC23766 inhibition of Rac activation, we subsequently demonstrated mobilization of HSC/P following a single *in vivo* dose of the compound.[25] Compared with phosphate-buffered saline (PBS)-treated controls, the Rac inhibitor induced an ~2-fold increase in circulating progenitors at 6 h after injection. The mobilization of HSC/P by NSC23766 was dose-dependent and reversible, with the number of circulating HSC/P returning to normal values within 24 h post injection. To determine if the effect of the inhibitor was associated with specific inhibition of Rac1 and Rac2, we incubated BM lineage negative/c-kit$^+$ cells with stromal-derived factor-1α and NSC23766. At a dose as low as 10 μM, activation of both Rac1 and Rac2 was inhibited.[25] We further found that NSC23766 was effective in suppressing downstream Rac signaling, as measured by the effect on the phosphorylation status of p21-activated kinase-1, a known effector of Rac. The inhibitor appeared to be both reversible and nontoxic *in vivo* when administered at a dose of 2.5 mg per kg daily for a period of 65 days.[25] Thus, NSC23766 constitutes a Rac-specific small molecule inhibitor that is capable of reversing cancer cell phenotypes associated with Rac deregulation and blocking Rac activation in HSC/P *in vivo*.

To validate Rac GTPases as antileukemic targets in p210-BCR-ABL-induced disease, we incubated p210-BCR-ABL-expressing murine cells with increasing concentrations of NSC23766.[20] NSC23766 potently inhibited the growth of these cells and also suppressed proliferation of Rac1/Rac2-deficient cells harvested from p210-BCR-ABL-expressing mice, further supporting the role of Rac3 hyperactivation in disease development. In addition, NSC23766 inhibited proliferation of cells expressing the tyrosine-kinase inhibitor-resistant p210-BCR-ABL-T315I mutation. In the presence of both NSC23766 and imatinib, proliferation of these cells was inhibited by >90%. Treatment with NSC23766 also led to selective killing of human CML blast crisis HSC/P with limited toxicity on normal murine or human HSC/P *in vitro*.

We then determined the effect of NSC23766 *in vivo* using our retroviral transduction and transplantation model. Mice in which NSC23766 was continually administered survived significantly longer than PBS-treated mice. NSC23766 reversed the CML survival/growth in an *in vivo* model of human CML disease after transplantation of chronic-phase purified CD34+ cells in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice.[20] These results further validate Rac as a candidate target in p210-BCR-ABL-mediated disease.

Could targeting Rac GTPases be useful for inducing leukemia stem cell egression from the nurturing leukemic stem cell niche?

Recently, the 'cancer stem cell' hypothesis, which attempts to explain the presence of 'residual' therapy-resistant cancer cells in patients and in related animal models, has attracted much attention.[53] The theory suggests that cancer may arise from a rare population of putative cancer stem cells. Leukemia stem cells (LSCs), which share characteristics with normal HSCs but initiate disease instead of supporting normal hematopoiesis, have been demonstrated in several leukemias, most prominently CML.[54] Like normal HSCs, LSCs are thought to reside in the BM niche, although the nature of LSC interaction with the supporting BM microenvironment, where leukemia presumably arises, remains unclear.[54] Recent studies in a human AML mouse model and a CML mouse model using anti-CD44 antibody to disrupt potential LSC interactions with the BM niche have provided strong evidence that LSCs depend upon interactions within a specific niche.[55,56] The fact that targeting the LSC-expressed cell surface molecule CD44 by monoclonal antibodies effectively suppressed both AML and CML leukemia progression, and induced LSC differentiation raises the possibility that the LSC–niche interaction could be a valid drug target for more effective eradication of leukemia. Since Rac activities are dysregulated in CML and Rac is known to be a central regulator of HSC adhesion, migration and interaction with the BM niche,[22,25] it is possible that Rac targeting by specific inhibitors could transiently mobilize LSCs for therapeutic benefits. As mentioned above, our group has shown previously that Rac1$^{-/-}$;Rac2$^{-/-}$ HSCs demonstrate defective adhesion, migration and lodging in the BM endosteum, and administration of the Rac activation inhibitor NSC23766 induces stem cell mobilization in a dose-dependent manner. It is thus an attractive proposal that future strategies of Rac targeting may be adopted in a similar fashion to induce egression of CML stem cells from the BM niche.

What type of CML patients may benefit from Rac inhibitor therapies?

Since multiple pathways, directly dependent or independent of BCR-ABL expression, are activated in CML during the evolution of disease, we postulate that Rac inhibition in conjunction with ABL tyrosine kinase domain inhibitors may represent a novel method of combined therapy early in the disease.

Levels of *BCR-ABL* mRNA,[57–59] protein[60] or phosphoprotein[61] increase during disease progression. This may be related to the higher levels of expression of BCR-ABL in CD34+ CML cells compared with more differentiated myeloid cells, and the fact that some patients even in complete cytogenetic response display a persistent population of HSC/P that express high levels of BCR-ABL.[62] High levels of expression of BCR/ABL are probably responsible for the fact that intracellular drug levels may be insufficient to reach the degree of kinase inhibition required to induce cell death in HSC/P.[63] Whether there is correlation between BAC-ABL expression and Rac activation is not yet known, but it is quite possible that a BCR-ABL gain of function translates into higher Rac activation. We found that Rac1, Rac2 and to a lesser extent, Rac3 are hyperactivated in CD34+ human chronic phase CML cells,[20] and a Rac-specific small molecule inhibitor significantly reduced the clonogenicity of blastic phase CML granulo-macrophage progenitors, and *in vivo* it significantly reduced the leukemic burden in NOD/SCID

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mice transplanted with chronic-phase CML CD34+ cells. Altogether this suggests that Rac inhibition may indeed impair leukemic growth in very different phases of the disease. This is even more relevant when chronic phase CML is believed to derive from HSC with engraftment and self-renewal ability, while blastic phase CML is believed not to derive from HSC but from granulo-macrophage progenitors.[64] The mechanisms of disease persistence in patients treated with tyrosine kinase inhibitors appear to be related to both BCR-ABL-dependent and -independent pathways.[65] Among the BCR-ABL-dependent pathways, the appearance of mutations in the kinase domain that confer high resistance (reviewed in Melo and Barnes[66]), or even moderate degrees of resistance to tyrosine kinase inhibitors[67] and the maintenance of high-expressing HSC/P even in patients in complete cytogenetic response have been shown to be responsible for disease persistence.

Among the BCR-ABL-independent pathways, the overexpression of drug transporters which are likely to influence intracellular levels of tyrosine kinase inhibitors and the simultaneous signaling through cytokine receptor-dependent pathways, in cells that still respond to cytokine stimulation, have been cited.[65] We are unable to confirm whether NSC23766 chemical conformation[51] is an adequate substrate for drug transporters, but since Rac activation is central in many of the key signaling pathways of HSC/P, Rac inhibition may also impair BCR-ABL-independent pathways and represent a valid adjuvant in the therapy of CML. In addition, since Rac appears likely to integrate multiple pathways downstream of BCR-ABL transforming activities, Rac inhibition combined with ABL tyrosine kinase domain inhibitors may represent a novel method of combined therapy early in the disease.

Summary and model of disease development

Rac GTPases appear to play a critical role in the development of leukemogenesis associated with p210-BCR-ABL expression and represent novel targets for therapeutic intervention, as depicted in the model shown in Figure 1. The p210-BCR-ABL fusion protein may activate Rac GTPases either directly through the Dbl homology domain of Bcr or via recruitment of the Vav1 GEF. In addition, loss of Rac expression may inhibit the formation of a large multimeric protein complex containing PI3K, DOK2, CrkL and Vav, thus inhibiting CrkL phosphorylation. Downregulation of Rac activation leads to almost complete abrogation of MAP kinase and PI3K signaling pathways, suggesting that Rac GTPases are required for activation of multiple p210-BCR-ABL-mediated signaling cascades.

Our results also suggest that STAT5 activation is maintained in the absence of Rac1 and Rac2. We speculate that Rac1/Rac2 activation may be a key for p210-BCR-ABL-induced leukemogenesis in this setting. While Rac1 and Rac2 isoforms activate multiple pathways, compensatory Rac3 activation may be responsible for signaling downstream of p210-BCR-ABL and inducing leukemogenesis in the absence of Rac1 and Rac2.

NSC23766, an inhibitor of all three isoforms of Rac that are expressed by hematopoietic cells, induces significant regression of murine p210-BCR-ABL-induced leukemias and human CML in xenogeneic grafts, demonstrating that intervention of Rac activation is a new tool in treating p210-BCR-ABL-induced leukemias. The finding that a single dose of the Rac inhibitor induces mobilization of HSC/P[25] raises the possibility that NSC23766 could also mobilize leukemic stem cells from their niche, thus inhibiting the stem cell properties characteristic of these cells. These results suggest that the Rac GTPases may prove to be useful therapeutically by targeting alternative signaling pathways, which may be responsible for resistance and relapse in CML.

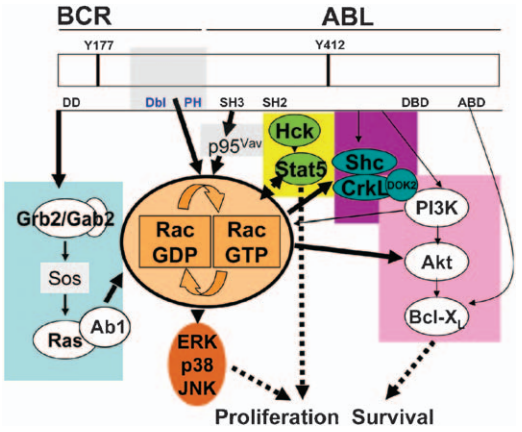

Figure 1 Model of activation of Rac GTPases in BCR-ABL-induced leukemogenesis. The various pathways of activation downstream of p210-BCR-ABL are indicated with different color codes. Gray-shaded areas indicate molecules or domains with guanine nucleotide exchange factor (GEF) activity. The Dbl and pleckstrin homology (Dbl and PH) domains, only present in p210-BCR-ABL, activate Rho GTPases directly. The SH3 domain in both p190- and p210-BCR-ABL activates p95Vav (Vav1). Y177/Y412, tyrosine residues that can be phosphorylated; Dbl, Rac GTPase exchange factor; SH2/SH3, Src homology domains; DD; dimerization domain; DBD, DNA-binding domain; ABD, actin-binding domain.

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SPOTLIGHT

CHAPTER TWENTY-SEVEN

Rho GTPases and Regulation of Hematopoietic Stem Cell Localization

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Abstract

Bone marrow engraftment in the context of hematopoietic stem cell and progenitor (HSC/P) transplantation is based on the ability of intravenously administered cells to lodge in the medullary cavity and be retained in the appropriate marrow space, a process referred to as homing. It is likely that homing is a multistep process, encompassing a sequence of highly regulated events that mimic the migration of leukocytes to inflammatory sites. In leukocyte biology, this process includes an initial phase of tethering and rolling of cells to the endothelium via E- and P-selectins, firm adhesion to the vessel wall via integrins that appear to be activated in an "inside-out" fashion, transendothelial

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migration, and chemotaxis through the extracellular matrix (ECM) to the inflammatory nidus. For HSC/P, the cells appear to migrate to the endosteal space of the bone marrow. A second phase of engraftment involves the subsequent interaction of specific HSC/P surface receptors, such as $\alpha_4\beta_1$ integrin receptors with vascular cell–cell adhesion molecule-1 and fibronectin in the ECM, and interactions with growth factors that are soluble, membrane, or matrix bound. We have utilized knockout and conditional knockout mouse lines generated by gene targeting to study the role of Rac1 and Rac2 in blood cell development and function. We have determined that Rac is activated via stimulation of CXCR4 by SDF-1, by adhesion via β_1 integrins, and via stimulation of c-kit by the stem cell factor—all of which involved in stem cell engraftment. Thus Rac proteins are key molecular switches of HSC/P engraftment and marrow retention. We have defined Rac proteins as key regulators of HSC/P cell function and delineated key unique and overlapping functions of these two highly related GTPases in a variety of primary hematopoietic cell lineages *in vitro* and *in vivo*. Further, we have begun to define the mechanisms by which each GTPase leads to specific functions in these cells. These studies have led to important new understanding of stem cell bone marrow retention and trafficking in the peripheral circulation and to the development of a novel small molecule inhibitor that can modulate stem cell functions, including adhesion, mobilization, and proliferation. This chapter describes the *biochemical footprint* of stem cell engraftment and marrow retention related to Rho GTPases. In addition, it reviews abnormalities of Rho GTPases implicated in human immunohematopoietic diseases and in leukemia/lymphoma.

1. Basic Mechanisms of Hematopoietic Stem Cell and Progenitor (HSC/P) Homing and Retention in Bone Marrow (BM)

Bone marrow engraftment in the context of HSC/P transplantation is based on the ability of intravenously administered cells to lodge in the medullary cavity and be retained in the appropriate marrow space, a process referred to as homing. It is likely that homing is a multistep process, encompassing a sequence of highly regulated events that mimic the migration of leukocytes to inflammatory sites. In leukocyte biology, this process includes an initial phase of tethering and rolling of cells to the endothelium via E- and P-selectins, firm adhesion to the vessel wall via integrins that appear to be activated in an ''inside-out'' fashion, transendothelial migration, and chemotaxis through the extracellular matrix (ECM) to the inflammatory nidus (Butcher and Picker, 1996; Peled *et al.*, 1999a; Springer, 1994). For HSC/P, the cells appear to migrate to the endosteal space of the bone marrow (Driessen *et al.*, 2003; Gong, 1978; Nilsson *et al.*, 2001; Wilson and Trumpp, 2006). A second phase of engraftment involves the

subsequent interaction of specific HSC/P surface receptors, such as $\alpha_4\beta_1$ integrin receptors with vascular cell–cell adhesion molecule-1 (VCAM-1) and fibronectin in the ECM, and interactions with growth factors that are soluble, membrane, or matrix bound (Williams *et al.*, 1991a,b). HSC/P can be temporarily detected in other organs such as liver, lung, and kidneys after intravenous infusion but disappear from these sites within 48 h after transplantation. In contrast, the retention of HSC/P in BM is sustained and appears specific (Papayannopoulou *et al.*, 2001a).

Some of the factors that influence this specific retention of HSC/P in the bone marrow have been defined recently and appear to involve the interplay among chemokines, growth factors, proteolytic enzymes, and adhesion molecules (Papayannopoulou, 2003).

Among the chemokines, stromal derived factor-1α (SDF-1α) and its receptor, the G-protein-coupled seven-span transmembrane receptor, CXCR4, play key roles in HSC trafficking and repopulation (Lapidot and Kollet, 2002). SDF-1α is expressed by both human and murine BM endothelium and stroma (Nagasawa *et al.*, 1998; Peled *et al.*, 1999a) and acts as a powerful chemoattractant of HSC/P (Aiuti *et al.*, 1997; Wright *et al.*, 2002). SDF-1α may also regulate the survival of HSC/P (Broxmeyer *et al.*, 2003; Lataillade *et al.*, 2000). SDF-1α induces the integrin-mediated firm arrest of hematopoietic progenitor cells and facilitates their transendothelial migration (Peled *et al.*, 1999a, 2000) and regulates HSC/P homing (Kollet *et al.*, 2001) and BM engraftment (Peled *et al.*, 1999b). Furthermore, SDF-1α is also required for the retention of murine HSC/P within the BM (Ma *et al.*, 1999; Nagasawa *et al.*, 1996). We have demonstrated that Rac proteins are activated by SDF-1 in HSC/P and that Rac-deficient HSC/P do not respond to SDF-1 (Fig. 27.1).

Among growth factors, a critical component for HSC/P survival and engraftment is the stem cell factor (SCF), which is expressed on BM stromal cells and is the ligand for the receptor tyrosine kinase, c-kit. A transmembrane isoform of SCF, membrane-bound SCF (membrane, mSCF), has been shown to be critical in the lodgment and retention of HSC within the hematopoietic microenvironment, although it does not appear to play a role in the homing of transplanted cells to BM (Driessen *et al.*, 2003). In addition, it appears that c-kit activation is differentially affected by soluble versus membrane-bound SCF and that mSCF appears to enhance maintenance of long-term hematopoiesis *in vitro* (Miyazawa *et al.*, 1995; Toksoz *et al.*, 1992) and induces overexpression of CXCR4 (Kollet *et al.*, 2001; Peled *et al.*, 1999b). Our studies of SCF-stimulated cell proliferation demonstrate that Rac activation is a critical component of c-kit signaling (see Fig. 27.1).

A third factor important for homing and engraftment of HSC/P are the integrin-mediated adhesion molecules. Among them, β_1 integrins are probably the best characterized, and inhibition of integrin function leads to defective medullary engraftment (Papayannopoulou and Craddock, 1997;

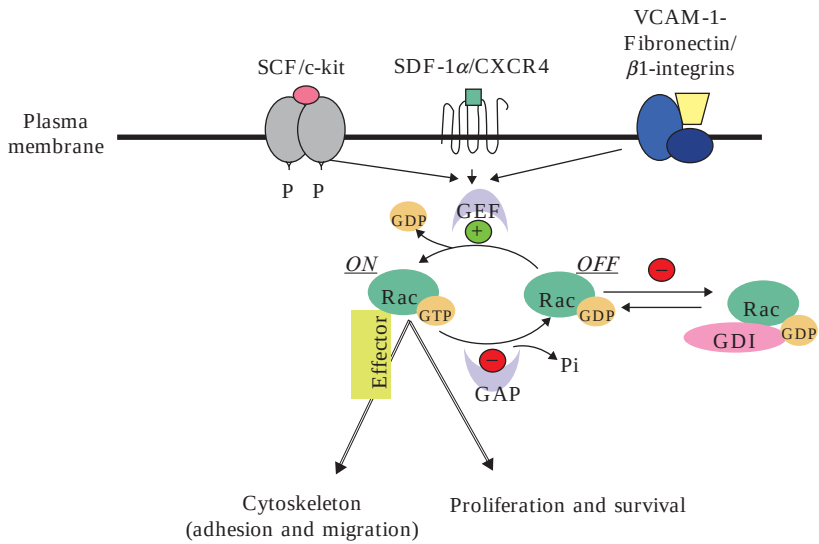

Figure 27.1 Rac GTPases integrate signals from multiple surface receptors involved in HSC engraftment and retention.

Papayannopoulou *et al.*, 1995, 2001a; Scott *et al.*, 2003; van der Loo *et al.*, 1998; Williams *et al.*, 1991a). As detailed later, Rac-deficient HSC/P show significantly defective adhesion to fibronectin (Cancelas *et al.*, 2005; Gu *et al.*, 2003; Yang *et al.*, 2001) (see Fig. 27.1).

2. Basic Mechanisms of HSC/P Mobilization and Trafficking

Hematopoietic stem cell and progenitor mobilization is also a dynamic and complex process (Kronenwett *et al.*, 2000; Rafii *et al.*, 2002; Thomas *et al.*, 2002). HSC/P must exit the stem cell niche in the BM (presumably; see comments later), migrate through the marrow sinusoidal endothelium, and gain access to the blood. Circulating HSC/P and BM-adherent HSC/P appear to be interchangeable. Studies utilizing parabiotic mice have demonstrated that HSC/P can leave their niche without induction, traffic through the bloodstream, and finally migrate into BM of the conjoined animal (Abkowitz *et al.*, 2003; Wang *et al.*, 2003; Warren *et al.*, 1960; Wright *et al.*, 2001). This suggests that HSC/P trafficking is a physiological process. If so, circulating HSC/P would be predicted to move into the BM microenvironment through transendothelial migration directed by chemoattractants and ultimately anchor within the extravascular BM space where

proliferation and differentiation occur. In this process, adhesion molecules, chemokine receptors, and integrin signaling require signal integration that drives cytoskeleton rearrangements and regulates gene expression, cell survival, and cell cycle activation. An additional proposed HSC location in the marrow is the vascular niche, where HSC would be attached to the fenestrated endothelium of the BM specialized vessels, so-called sinusoids (Kiel *et al.*, 2005). Such an outlook is supported by evidence that Rac-deficient HSC with profoundly defective cell migration due to loss of the combined function of CXCR4, β_1 integrins, and c-kit signaling pathways can be mobilized in large numbers (Cancelas *et al.*, 2005) [for a commentary, see Cancelas *et al.* (2006)]. The BM sinusoids express molecules important for HSC mobilization, homing, and engraftment, including chemokines such as CXCL12 (ligand for CXCR4) and adhesion molecules such as endothelial-cell (E)-selectin and vascular cell-adhesion molecule 1 (VCAM-1). These findings have given additional microanatomical clarity to the concept of stem cell niches as spatial structures in which HSC reside, self-renew, and differentiate.

At the molecular level, the interaction between SDF-1α and the G-coupled chemokine receptor CXCR4 has been recognized as pivotal in stem cell mobilization. As HSC/P are known to migrate toward a SDF-1α (Sweeney *et al.*, 2002), it has been suggested that treatment with granulocyte colony-stimulating factor-1 (G-CSF), cyclophosphamide, or interleukin (IL)-8 leads to a reduction of SDF-1β in BM, resulting in a positive gradient in blood and induction of HSC/P migration toward PB. Raising the plasma levels of SDF-1α by intravenous injection of SDF-1α-expressing adenovirus (Hattori *et al.*, 2001) or sulfated polysaccharides (Sweeney *et al.*, 2002) or by inhibition of the CXCR4 receptor (Devine *et al.*, 2004; Liles *et al.*, 2003; Tavor *et al.*, 2004) leads to mobilization of HSC/P. G-protein inhibition by pertussis toxin (Papayannopoulou *et al.*, 2003) induces a similar mobilization effect, probably by interfering with the CXCR4 signaling pathway. It has been suggested that bone expression of CXCL12 (another ligand for CXCR4) is regulated by G-CSF-induced β_2-adrenergic signals that modify osteoblast protein expression and shape (Katayama *et al.*, 2006).

Functional blocking of $\alpha_4\beta_1$ integrin (receptor for VCAM-1 and fibronectin) alone or together with $\alpha_l\beta_2$ integrins or the functional blocking of the β_2 integrin leukocyte function-associated antigen-1 by antibodies results in the mobilization of HSC/P (Craddock *et al.*, 1997; Papayannopoulou *et al.*, 2001b). HSC/P accumulate in the PB soon after gene deletion in inducible $\alpha_4\beta_1$ integrin-deficient mice. Although their numbers gradually stabilize at a lower level, progenitor cell influx into the circulation continues at above-normal levels for more than 50 weeks with a concomitant progressive accumulation of spleen HSC/P (Scott *et al.*, 2003).

Playing an important and independent role in HSC/P mobilization is the interaction between SCF and its receptor, c-kit. SCF/c-kit interaction

plays a critical role in G-CSF-mediated mobilization (Heissig *et al.*, 2002; Levesque *et al.*, 2003), and SCF in combination with G-CSF has been shown to enhance HSC/P mobilization (McNiece and Briddell, 1995). As mentioned earlier, tm-SCF has been shown to be critical in retaining HSC/P in BM (Driessen *et al.*, 2003).

3. Rho GTPases

Almost all Rho family GTPases influence actin polymerization within the cell via specific or shared effectors and are thereby implicated in reorganization of the cytoskeleton, migration, and adhesion. However, Rho proteins regulate a multitude of other cellular functions. Among these are apoptosis and survival, cell cycle progression, and genomic stability. The activity of individual Rho proteins can be regulated by multiple guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs), which are cell and agonist specific. Indeed, Rho GTPases such as Rac appear to integrate signaling from multiple receptors in individual cells. For instance, as mentioned earlier and detailed later, we have shown in hematopoietic cells that Rac is activated by stimulation of CXCR4 via SDF-1, adhesion via β_1 integrins, and stimulation of c-kit via SCF—all pathways involved in stem cell engraftment (Cancelas *et al.*, 2005; Gu *et al.*, 2003; Yang *et al.*, 2001). In addition, Rho family members recognize both unique and shared effectors. This, at least in part, explains the diversity of cellular functions influenced by a single Rho GTPase but also presents significant complexities in developing an understanding of the physiological roles of these proteins, particularly if studies utilize cell lines and expression of dominant negative (DN) or constitutive active (CA) mutants, which generally lack specificity among related GTPases. We have exploited mouse knockouts to study the function of Rac GTPases in hematopoiesis in an attempt to circumvent the problems associated with these DN and CA mutants.

Using genetic approaches (primarily gene targeting in mice), we have specifically implicated PAK, POR1, and STAT5 in Rac effector functions in primary hematopoietic cells and, depending on the specific lineage and agonist, found that Rac can activate p42/p44 and p38 ERKs, JNK, and Akt kinases (Cancelas *et al.*, 2005; Carstanjen *et al.*, 2005; Gu *et al.*, 2003; Roberts *et al.*, 1999; Yang *et al.*, 2000, 2001) (and preliminary data). In a similar manner to the Wiskott–Aldrich syndrome protein (WASp), a key downstream target of Rac, WAVE1/2 and insulin receptor substrate (IRS) p53, has been implicated by others (primarily Takenawa and Miki, 2001) in actin polymerization and assembly. IRSp53 is a linker between Rac and WAVE1/2 that adds specificity for the actin-related protein (Arp)2/3

complex activator in actin polymerization. WAVE1 is required for Rac-mediated dorsal membrane ruffling, whereas WAVE2 may be involved in Rac-induced peripheral ruffling during cell migration (Miki *et al.*, 2000; Suetsugu *et al.*, 2003). The induction of actin polymerization by WAVE is dependent on Arp2/3 via the VCA (verprolin homology, cofilin homology, acidic) domain of WAVE1/2 in a manner analogous to WASp. The VCA domain is a G-actin and Arp2/3-binding domain required for Rac/Cdc42-induced *de novo* actin nucleation and actin polymerization (reviewed in Takenawa and Miki, 2001). While the physiological relevance of these molecular links to Rac remains unknown, *de novo* actin nucleation appears critical for actin assembly at the leading edge of migrating cells and we hypothesize that this is of particular relevance to the migration of hematopoietic cells. In addition (and likely also relevant to the BM microenvironment), WAVE is essential for cell migration mediated through ECM in mouse embryo fibroblasts (Eden *et al.*, 2002).

Thus, the combination of unique and shared upstream activators and downstream effectors, which may be cell type specific, represents an important mechanism by which the same Rho GTPase regulates a variety of the aforementioned cellular processes. The utilization of a genetic approach and the study of primary cells in murine models have contributed greatly to a new understanding of both unique and overlapping functions of Rho GTPases in hematopoiesis.

A significant proportion of known Rho GTPases is expressed ubiquitously, while two Rho proteins show tissue-specific expression. This is particularly important in hematopoietic cells and has been studied extensively for the Rac subfamily. All members of this subfamily (Rac1, Rac2, and Rac3) show high sequence similarity. Rac1 is expressed ubiquitously, whereas Rac3 is expressed in nearly all cell lines examined thus far and is expressed at high levels in murine heart, placenta, pancreas, and brain. Rac2 is expressed in a hematopoietic-restricted fashion. Thus, hematopoietic cells are unique in that all three Rac proteins are coexpressed and also express RhoH, the only other identified hematopoietic-specific Rho GTPase. RhoH has been shown by us and others to modulate Rac signaling (Gu *et al.*, 2005b, 2006; Li *et al.*, 2002a). Despite this expression pattern and their significant homology, individual Rac proteins are responsible for unique functions in hematopoietic cells, as gene-targeted mice deficient in each protein have measurable and distinct phenotypes. This has been well documented by our group and others using gene-targeted mice to examine the role of Rac1 versus Rac2 in hematopoietic cells (Fig. 27.2) (and see later). Genetic deletion of Rac2 leads to a number of phenotypic changes in multiple hematopoietic lineages, including granulocytes (Abdel-Latif *et al.*, 2004; Carstanjen *et al.*, 2005; Filippi *et al.*, 2004; Glogauer *et al.*, 2003; Kim and Dinauer, 2001; Lacy *et al.*, 2003; Li *et al.*, 2002a; Roberts *et al.*, 1999), B cells (Croker *et al.*, 2002b; Walmsley *et al.*, 2003), T cells (Croker *et al.*, 2002a),

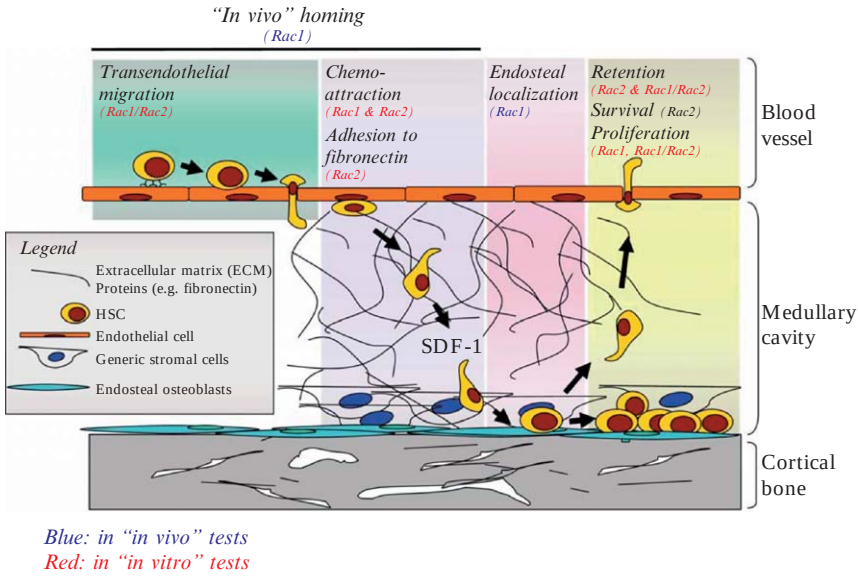

Figure 27.2 **Unique and overlapping roles of Rac1 versus Rac2 in hematopoiesis. Putative utilization of Rac1 and Rac2 in processes involved in homing, migration, endosteal localization, retention, and proliferation/survival is shown.**

mast cells (Gu *et al.*, 2002; Tan *et al.*, 2003; Yang *et al.*, 2003), eosinophils (Fulkerson *et al.*, 2005), and platelets (Akbar *et al.*, 2006), despite continued expression (and even a compensatory increase in expression in some cases) of Rac1. Considerable evidence shows that Rac2 and Rac1 regulate both separable and overlapping functions in nearly all lineages on the hematopoietic cells examined (Filippi *et al.*, 2004; Gu *et al.*, 2003; Walmsley *et al.*, 2003). Thus, Rac1 cannot compensate for the loss of Rac2 function in hematopoietic cells and vice versa.

In addition, Rho GTPases from different subgroups appear to demonstrate cross talk to regulate cellular responses. In previous studies, primarily in fibroblasts, introduction of constitutive active or dominant negative mutants of Cdc42, Rac, and RhoA was shown to affect activation or inhibition of each other. Growth factor receptor-induced activation of Cdc42 has been shown to activate Rac, which in turn stimulates Rho activity, resulting in cytoskeletal remodeling. RhoE has been shown to downregulate the activity of RhoA by activating p190RhoGAP. Similarly, as mentioned earlier, RhoH has been shown to repress Rac activity in lymphoid cell lines (Li *et al.*, 2002a) and cytokine-stimulated hematopoietic progenitor cells, resulting in reduced proliferation, increased apoptosis, and defective actin polymerization (Gu *et al.*, 2005a). In addition, we have demonstrated that expression of a patient-derived dominant negative Rac

GTPase, D57N, in hematopoietic cells not only inhibits Rac1 and Rac2 activities, but may also inhibit Cdc42 (Y. Gu and D. Williams, unpublished results). Additional genetic studies have demonstrated cross-talk between Rac and both Cdc42 (Yang *et al.*, 2001) and RhoA (Filippi *et al.*, manuscript in preparation).

4. Role of Rac1 and Rac2 GTPases in Hematopoiesis

Using gene-targeted mice, it has become evident that the Rho family of GTPases plays an important role in hematopoietic stem cell function. Rac activity has been demonstrated to be important for such diverse functions as retention in the bone marrow (Cancelas *et al.*, 2005; Yang *et al.*, 2001), long-term engraftment of HSC (Jansen *et al.*, 2005), and HSC mobilization (Gu *et al.*, 2003). Furthermore, in more committed hematopoietic cells, Rac activity is associated with B-lymphocyte development and signaling (Croker *et al.*, 2002b; Walmsley *et al.*, 2003), granulocyte chemotaxis and superoxide production (Abdel-Latif *et al.*, 2004; Carstanjen *et al.*, 2005; Filippi *et al.*, 2004; Glogauer *et al.*, 2003; Kim and Dinauer, 2001; Lacy *et al.*, 2003; Li *et al.*, 2002a; Roberts *et al.*, 1999), migration and degranulation of mast cells (Gu *et al.*, 2002; Yang *et al.*, 2000), differentiation of mature osteoblasts (Lax *et al.*, 2004), and maturation of TRAP-positive, pro-osteoclasts into multinucleated osteoclasts (Korhonen *et al.*, manuscript in preparation).

Rac1-deficient HSC/P stimulated with SCF demonstrate defective proliferative signaling from the c-kit receptor tyrosine kinase *in vitro* (Gu *et al.*, 2003). In contrast, loss of Rac2 activity leads to a pro-apoptotic phenotype in both mast cells and HSC/P in the presence of SCF (Gu *et al.*, 2002, 2003; Yang *et al.*, 2000). Rac integrates signals from β_1 and β_2 integrins and c-kit in HSC/P and mast cells (Gu *et al.*, 2002, 2003; Tan *et al.*, 2003). Signaling of c-kit to Rac is mediated through the GEF Vav (see later and preliminary data), although the specific Vav responsible for signaling to Rac in nonlymphoid hematopoietic cells remains largely unknown. Thus, overall, studies utilizing mouse mutants implicate Rac proteins downstream of CXCR4, c-kit, and β_1 and β_2 integrins, and Rac-deficient hematopoietic cells show loss of adhesion, migration, degranulation, changes in cell shape consistent with deregulated actin assembly, and defects in cell proliferation and survival linked to alterations in kinase pathways that are both lineage and agonist specific (for a complete review, see Cancelas *et al.*, 2006). Rac GTPases are thus important molecular switches controlling stem cell localization and retention in the marrow microenvironment, engraftment, and reconstitution in transplanted mice. These

proteins represent a novel molecular target to modulate hematopoietic cell functions (Nasser *et al.*, 2006), and we have developed a first-generation, small molecule inhibitor, NSC23766, which induces mobilization of HSC/P (Cancelas *et al.*, 2006).

5. Rac3 GTPase, a Newly Defined Member of the Rac Family Cloned from a Bcr-abl Transformed Cell Line

Rac3 is a third member of the Rac subfamily, which was originally identified from a chronic myelogenous leukemia cell line, and has been implicated in human breast cancer (Baugher *et al.*, 2005; Mira *et al.*, 2000), ovarian cancer (Morris *et al.*, 2000), cellular transformation (Keller *et al.*, 2005), and tumor invasion (Chan *et al.*, 2005). Rac3 has been shown to interact with the integrin-binding protein CIB and promotes integrin-mediated adhesion and spreading in immortalized cell lines. In addition, Rac3 has been shown to be expressed differentially during myeloid differentiation (U. Knaus, personal communication). Rac3 null mutant mice have been reported (Cho *et al.*, 2005; Corbetta *et al.*, 2005) and are viable, fertile, and without obvious physical anomalies. One group has reported a mild neurological phenotype (Corbetta *et al.*, 2005). In addition, in a p190 Bcr-abl transgenic mouse model of acute lymphoblastic leukemia, Rac3, but not Rac1or Rac2, is activated and Rac3 deficiency attenuates the development of leukemia in female mice (Cho *et al.*, 2005). Data suggest that in the absence of Rac1 and Rac2, Rac3 can mediate an attenuated myelodysplastic phenotype in mice transplanted with p210 Bcr-abl (see preliminary data). However, no systematic analysis of hematopoiesis has been reported in Rac3-/- mice. Using reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot analysis, Burkhalter and co-workers (2002) have reported that the expression of Rac3 expression is downregulated dramatically during terminal myeloid differentiation. The functional significance of this observation has not been reported. We have demonstrated normal neutrophil differentiation in Rac1-/-; Rac2-/- cells (Filippi *et al.*, 2004; Gu *et al.*, 2003), but observed abnormal myeloid development *in vitro* after transduction of HSC/P with the dominant negative D57NRac2, which most likely inhibits Rac3 in addition to Rac1 and Rac2 (Tao *et al.*, 2002). These data suggest that Rac3 may be important in myelopoiesis, but suffer from the weaknesses of the use of DN mutants, the effects of which are not specific and are determined in part by expression levels. Interestingly, HSC/P expressing D57NRac2 fail to reconstitute hematopoiesis when transplanted into lethally irradiated recipients (Gu *et al.*, 2002).

6. Cdc42 in Hematopoiesis

Cdc42 has been linked for some time with gradient sensing and filopodia (Ridley and Hall, 1992). Until very recently, most work in hematopoietic cells utilized macrophage cell lines or examined the role of Cdc42 in lymphocytes, and interest in lymphocytes is derived in part because of the association of the Cdc42 target WASp in the human immunodeficiency disease of the same name (Symons *et al.*, 1996). In macrophages and myeloid cell lines, DN Cdc42 expression or Cdc42 inhibition in cell lines is associated with a lack of polarization in response to the growth factor/chemotactic factor CSF-1, leading to reduced directed but not random migration (Allen *et al.*, 1998; Srinivasan *et al.*, 2003). In monocytes, either constitutive active or DN Cdc42 expression leads to reduced migration across the endothelium (Weber *et al.*, 1998). In primary T cells, DN Cdc42 reduces chemotaxis in response to SDF-1, a potent chemokine for lymphocytes (del Pozo *et al.*, 1999).

More recent studies have utilized gene-targeted mice. Loss of the Cdc42 GEF PIXα leads to defective G-coupled receptor signaling and PAK activation and reduced migration. Gene-targeted mice deficient in the Cdc42 GAP protein exhibit increased Cdc42 activity in the bone marrow with increased apoptosis in HSC populations. Hematopoietic cells exhibit disorganized actin structure and defective engraftment in stem cell transplant protocols (Wang *et al.*, 2006). Neutrophils from gene-targeted Cdc42-deficient mice show increased random motility but reduced directed migration associated with reduced podosome-like structures at the leading edge of the cells (Szczur *et al.*, 2006). Cdc42-/- neutrophils show increased lateral and tail membrane protrusions. Directed migration appears inhibited by defective p38MAPK activity apparently required for antagonizing these lateral filopodia-like structures. HSC from Cdc42-/- mice show defective migration and adhesion, which is associated with abnormal F-actin assembly, homing, and engraftment/retention in the bone marrow. Cdc42-/- mice show increased numbers of circulating HSC and reduced development of erythrocytes with anemia (Yang *et al.*, 2007a). In contrast to Cdc42 GAP-/- mice, these animals do not show increased apoptosis, but do show abnormalities in cell cycle progression associated with dysregulated p21 and cMyc expression. More recent studies show that Cdc42 regulates the balance between myelopoiesis and erythropoiesis (Yang *et al.*, 2007b). Cdc42-deficient mice developed a fatal myeloproliferative disorder characterized by neutrophilia, myeloid cell proliferation, and infiltration into multiple organs. Early erythroid development was inhibited. Bone marrow of Cdc42-/- mice showed decreased erythroid burst-forming units and erythroid colony-forming units. These changes were associated with upregulation of the

myeloid transcription factor PU.1, C/EBP1α, and Gfi-1 and downregulation of GATA-2.

7. RhoA in Hematopoiesis

The effect of RhoA on hematopoiesis has been less well studied compared with Rac and Cdc42. As noted previously, activation of RhoA leads to stress fiber formation and cell shape changes, although most of these studies have been performed on fibroblasts. In fibroblasts, activation of RhoA has been reported to decrease the expression of Cdk inhibitors and to shorten G1 (Olson *et al.*, 1998). Using the same cell types, inactivation of RhoA has been shown to induce the expression of cyclin D–Cdk4 complexes in early G1 phase and promote a rapid G1/S phase transition (Roovers *et al.*, 2003; Welsh *et al.*, 2001). In mammary gland epithelial cells, transforming growth factor-β-induced activation of RhoA stimulates the nuclear translocation of p160 ROCK, a known target of RhoA, which results in cell cycle arrest by decreasing the activity of Cdc25A phosphatase and decreasing Rb phosphorylation (Bhowmick *et al.*, 2003). Therefore, the effect of RhoA GTPase activity on cell cycle and proliferation appears both cell type and agonist specific.

We have examined the role of RhoA GTPase in hematopoietic stem and progenitor cell functions by expressing DN mutant RhoAN19 in HSC/P via retrovirus-mediated gene transfer (Ghiaur *et al.*, 2006). In contrast with the published role of RhoA in fate determination and differentiation in mesenchymal stem cells (Sordella *et al.*, 2003), inhibition of RhoA activity was associated with a significant enhancement of HSC engraftment and reconstitution *in vivo*. Increased engraftment of HSC expressing RhoAN19 was associated with increased cyclin D1 expression and enhanced proliferation and cell cycle progression of hematopoietic progenitor cells *in vitro*, despite this enhanced engraftment *in vivo*. Consistent with studies reported in fibroblast cells (Hall, 1998), RhoA was essential for normal adhesion and migration of hematopoietic progenitor cells *in vitro*. Decreased activity of RhoA GTPase resulted in defective $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin-mediated adhesion and impaired SDF-1α-directed migration of hematopoietic progenitor cells *in vitro*. These results are surprising given the role of adhesion and migration in HSC engraftment. Taken together, these data suggest that RhoA GTPase plays a crucial role in HSC engraftment, although the mechanism of enhanced engraftment seen with expression of the DN RhoA protein is unclear. In the context of previous reports describing Rac GTPase function in HSC (Cancelas *et al.*, 2005; Gu *et al.*, 2003), these studies suggest that inhibition in Rac activity may enhance mobilization, whereas inhibition of RhoA may augment HSC engraftment.

Additional studies using gene-targeted mice are needed to better clarify the role of RhoA, RhoB, and RhoC in hematopoiesis.

8. RhoGTPase in Human Diseases

8.1. Rac deficiency syndrome

The initial report of Rac2-deficient mice described a phagocytic immunodeficiency syndrome emphasizing neutrophil dysfunctions related to actin cytoskeletal abnormalities. Subsequently, Ambruso *et al.* (2000) and our own group (Williams *et al.*, 2000) reported the identification of a child with serious, life-threatening infections associated with a dominant negative mutation of Rac2 (D57N). The patient exhibited leukocytosis but reduced inflammatory infiltrate in areas of infection. Neutrophils from this patient responded normally with respect to the respiratory burst to phorbol 12-myristate 13-acetate. Normal expression of CD11b, CD11c, and CD18 suggested that the patient did not suffer from leukocyte adhesion deficiency (LAD) or classical chronic granulomatous disease (CGD). However, the patient's neutrophils exhibited decreased chemotaxis in response to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukin-8, reduced rolling on GlyCAM-1 (a ligand for L-selectin), reduced superoxide generation in response to fMLP, mildly reduced phagocytosis, and adhesion to fibrinogen. Thus, the patient appeared clinically to have a phenotype overlapping between LAD and CGD.

At the molecular level, both genomic and cDNA sequencing confirmed the presence of the Asp→Asn mutation at position 57. Genomic sequencing confirmed a mono-allelic change in the gene, and ≈50% of the cloned cDNAs exhibited this mutation. Expression of the mutant protein via retrovirus-mediated gene transfer in normal neutrophils reproduced the cellular phenotype. The mutant protein displayed 10% GTP-binding activity, resulting in a markedly enhanced rate of GTP dissociation and did not respond to GEFs (Gu *et al.*, 2001). When expressed in murine-derived HSC, D57N Rac2 reduced endogenous activities of both Rac1 and Rac2 and led to decreased cell expansion *in vitro* associated with increased apoptosis. Transplantation of transduced bone marrow into lethally irradiated recipients showed a markedly reduced reconstitution of hematopoiesis in mutant-expressing cells over time, consistent with the role of Rac GTPases in marrow engraftment and retention described earlier. Interestingly, prior to successful curative allogeneic transplantation of this patient, his peripheral blood counts were diminishing and he was mildly pancytopenic at the time of marrow ablation in preparation for the transplant.

Taken together, these data suggest that the mutation behaves as a dominant negative mutation, likely by sequestering multiple GEFs in the cell.

This is a highly conserved amino acid in all GTPases and in the Ras superfamily and coordinates the binding of the γ-phosphate to the GTPase. Addition of recombinant Rac2 to cell-free extracts from the patient's neutrophils restored superoxide production, demonstrating the specificity of the molecular mutation in Rac2. This single case is the first reported mutation in humans of a GTPase and provides a fascinating correlation between the basic biology as elucidated in gene targeting models and human disease phenotype. Undoubtedly additional similar cases will become apparent, as many children with recurrent infections and neutrophil dysfunction remain poorly characterized at the molecular level.

8.2. Wiskott–Aldrich disease

The Cdc42 effector protein WASp is defective in the X-linked immunodeficiency disorder Wiskott–Aldrich syndrome (WAS) (Ochs and Thrasher, 2006; Thrasher and Burns, 1999). WASp activation depends on the specific interaction with guanosine triphosphate (GTP)-loaded Cdc42, which is mediated through a Cdc42- and Rac-interactive binding (CRIB) domain (Abdul-Manan *et al.*, 1999; Miki *et al.*, 1998; Rohatgi *et al.*, 1999). WASp is expressed in a hematopoietic-specific fashion. A spectrum of clinical disease is seen that correlates with mutations in specific domains of the WASp protein. Classic WAS patients express no WASp and have severely defective immune function that is characterized by aberrant polarization and directed migration of hematopoietic cells. WASp and the isoform N-WASp activate Arp 2/3, which regulates polymerization of actin from the barbed and branching filaments. WASp-deficient mice have been generated and also show significant hematopoietic defects.

Macrophages and dendritic cells from patients with WAS and from WASp-deficient mice have been shown to be defective in their migratory behavior. Chemotaxis of mutant macrophages in response to macrophage colony-stimulating factor (M-CSF), fMLP, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1a (MIP1 a) has been shown to be abrogated (Badolato *et al.*, 1998; Zicha *et al.*, 1998). WASp-deficient dendritric cells (DCs) exhibit similar abnormalities of cytoskeletal organization, chemotaxis, and migration (Binks *et al.*, 1998). WASp-deficient murine DCs exhibit multiple defects of trafficking *in vivo* after stimulation, including the emigration of Langerhans cells from the skin to secondary lymphoid tissues and the correct localization of DCs within T-cell areas, which correlated with a deficient migratory response of dendritic cells to the chemokines CCL19 and CCL21 (de Noronha *et al.*, 2005; Snapper *et al.*, 2005). It is therefore possible that DC trafficking abnormalities contribute in a significant way to the immune dysregulation observed in WAS and are responsible for the inflammation initiation and eczema development in this disease.

Defects of migration, anchorage, and localization have been defined more recently for other cell lineages, including T and B lymphocytes, neutrophils, and HSC/P. T lymphocytes from patients with WAS respond less well than normal cells *in vitro* to CXCL12 and CCL19 and demonstrate abrogated homing to secondary lymphoid tissue after adoptive transfer *in vivo* (Haddad *et al.*, 2001; Snapper *et al.*, 2005). There is a defect in the localization and function of the immunologic synapse, as WASp is recruited to lipid rafts immediately after the T-cell receptor and CD28 triggering event and is required for the movements of lipid rafts. T cells from WAS patients, lacking WASp, proliferate poorly after TCR/CD28 activation and have impaired capacities to cluster the lipid raft marker GM1 and to upregulate GM1 cell surface expression (Dupre *et al.*, 2002). Interestingly, cells that are deficient for both WASp and Wiskott–Aldrich syndrome protein-interacting protein (WIP) exhibit much more profound deficiencies than either alone, suggesting the existence of some redundancy (Gallego *et al.*, 2006). Similarly, WASp-deficient B lymphocytes have been shown to have marked morphologic abnormalities, defective migration, and adhesion *in vitro* and impaired homing *in vivo* (Westerberg *et al.*, 2005). This defect is likely to contribute to the observed deficiencies of humoral responses to both T-dependent and T-independent antigens and to the marked deficiency of marginal zone B cells in both murine and human spleens (Facchetti *et al.*, 1998; Westerberg *et al.*, 2005). A further example of defective trafficking *in vivo* originates from the observation that carrier female subjects for classic WAS almost universally exhibit nonrandom X-inactivation patterns in $CD34^+$ bone marrow progenitors (Wengler *et al.*, 1995). This implies that WASp is functional within the HSC/P compartment and is consistent with evidence for WASp expression in this cell type in human adult and embryonic hematopoietic stem cells (Marshall *et al.*, 2000; Parolini *et al.*, 1997). Serial stem cell transplantation and competitive repopulation studies in mice have confirmed a selective homing and engraftment advantage for normal HSC, and hematopoiesis established by means of engraftment of chimeric fetal liver populations results in dominance of normal HSC/P over WASp-deficient hematopoiesis (Lacout *et al.*, 2003), suggesting that throughout development, there be preferential establishment of hematopoiesis by normal rather than mutant HSCs due to an intrinsic homing advantage.

Much can be learned from the study of human patients with naturally occurring mutations. Most molecular defects in the WASp gene result in diminished activity, either because aborted protein production or because of intrinsic instability the mutant mRNA or protein. However, some mutants have been shown to display impaired interaction with key regulators. For instance, patients with X-linked thrombocytopenia express lower levels of WASp and have residual immune function (Lemahieu *et al.*, 1999). Some WASp gene defects result in expression of mutant protein with amino

acid substitutions within the Ena/VAS homology 1 domain, predictive of a disturbed interaction with WIP (Volkman *et al.*, 2002). Some clinically relevant mutations have been shown to abolish *in vivo* proper N-WASp localization and actin polymerization (Moreau *et al.*, 2000). Some X-linked neutropenia patients have missense mutations in the Cdc42-binding site of the WASp protein, and a subset of these patients has activating mutations that lead to constitutive activation of the protein. These activating mutations act by preventing autoinhibition of the Cdc42-binding domain of the molecule inducing unregulated actin polymerization and abnormal cytoskeletal structure and dynamics (Ancliff *et al.*, 2006). Interestingly, the phenotype of clinical disease arising from these mutations affecting the Cdc42-binding site is quite unlike that of classical WAS. These mutations lead to myelodysplastic changes in the bone marrow, reduced lymphocyte numbers and function, and increased apoptosis in the myeloid lineage associated with neutropenia and markedly abnormal cytoskeletal structure and dynamics. The mechanism of this defect is unclear but it can be related to abnormalities of cytokinesis affecting the chromosomal separation during mitosis.

8.3. Rac hyperactivation in leukemia

Rac GTPases have been previously implicated in p210-BCR-ABL-mediated transformation (Burridge and Wennerberg, 2004; Harnois *et al.*, 2003; Renshaw *et al.*, 1996; Schwartz, 2004; Sini *et al.*, 2004; Skorski *et al.*, 1998), although the specific role(s) of individual Rac subfamily members in the development of disease *in vivo* has not been defined. Evidence also suggests that Rac3 plays a role in p190-BCR-ABL-mediated ALL, whereas Rac1 and Rac2 do not appear to be hyperactivated in these lymphoma lysates (Cho *et al.*, 2005). This is of particular relevance, as p190-BCR-ABL differs from p210 in potentially important ways as it relates to RhoGTPases. For instance, while p210-BCR-ABL binds to and activates the Rho GTPases, apparently through the Dbl homology domain, p190-BCR-ABL, which lacks this domain, cannot bind to Rho GTPases but can still activate Rac1 and Cdc42 (Harnois *et al.*, 2003) through activation of the GEF Vav1 by BCR-ABL (Bassermann *et al.*, 2002). Rac GTPases have been shown to regulate signaling pathways that are downstream of p210-BCR-ABL (Burridge and Wennerberg, 2004; Schwartz, 2004). Together, these data suggest that Rac GTPases may integrate multiple signaling components of p210-BCR-ABL-activated pathways.

We analyzed whether Rac isoforms were hyperactivated in human chronic phase CML HSC/P. Activation of Rac was determined by p21-activated kinase (PAK) binding domain pull-down assays in isolated $CD34^+$ cells from CML patients. We observed that Rac1, Rac2, and, to a lesser degree, Rac3 were hyperactivated in $CD34^+$ cells purified from peripheral

blood of two CML patients at diagnosis (Thomas *et al.*, 2007). We subsequently utilized a retroviral murine model in Rac gene-targeted BM cells to investigate the importance of Rac GTPase activation in the development and progression of p210-BCR-ABL-mediated MPD. We showed that the combined deficiency of Rac1 and Rac2 significantly attenuates p210-BCR-ABL-induced proliferation *in vitro* and MPD *in vivo*. Attenuation of the disease phenotype is associated with severely diminished p210-BCR-ABL-induced downstream signaling in primary hematopoietic cells. These data are consistent with previous reports of Rac3 activation in p190-BCR-ABL expressing malignant precursor B-lineage lymphoid cells (Cho *et al.*, 2005). We then utilized NSC23766, a small molecule antagonist of Rac activation (Gao *et al.*, 2004), to biochemically and functionally validate Rac as a molecular target in both a relevant animal model and in primary human CML cells *in vitro* and in a xenograft model *in vivo*, including in imatinib-resistant p210-BCR-ABL disease. These data demonstrate that Rac is an important signaling molecule in BCR-ABL-induced transformation and an additional therapeutic target in p210-BCR-ABL-mediated myeloproliferative disease. Additional studies in other chronic and acute myelogenous leukemia may define the role of Rac and other GTPases in both chronic and acute leukemias.

8.4. RhoH and lymphomas

The RhoH/TTF (Translocation Three Four) gene was first identified as a fusion protein containing the LAZ3/BCL6 oncogene as a result of the t(3;4)(q27;p11) translocation in a non-Hodgkin's lymphoma (NHL) cell line (Dallery *et al.*, 1995; Dallery-Prudhomme *et al.*, 1997). A chromosomal alteration involving the RhoH/TTF gene in the t(4;14)(p13;q32) translocation has also been found in another patient with multiple myeloma (Preudhomme *et al.*, 2000). In some cases, RT-PCR analyses of NHL patients have shown deregulated expression of both RhoH and BCL6 genes by promoter exchange between these two genes (Preudhomme *et al.*, 2000). The RhoH gene, along with three other oncogenes (PIM1, MYC, and PAX5), has been found to have a more than 45% mutation rate in human diffuse large B-cell lymphomas (DLBCLs) (Pasqualucci *et al.*, 2001). Mapping analyses demonstrated mutations scattered throughout the 1.6 kb of intron 1 in the RhoH gene in 13 of 28 DLBCLs, suggesting potential effects on the regulation of RhoH gene expression with pathophysiological relevance. Similar aberrant hypermutation in the RhoH gene also occurs in AIDS-related non-Hodgkin lymphomas (Gaidano *et al.*, 2003) and primary central nervous system lymphomas (Montesinos-Rongen *et al.*, 2004). However, it remains unclear whether these mutations translate into abnormal levels of RhoH expression in lymphomas and what physiological contribution hypermutation in the RhoH gene plays in

lymphomagenesis. p53, a tumor suppressor gene, is a key regulator of apoptosis and cell cycle arrest upon DNA damage in many cells. p53 is the most frequently altered tumor suppressor in human solid tumors and is also altered in hematologic malignancies. Interestingly, p53 inactivation is frequent in transformed follicular lymphomas (80%) (Lo Coco *et al.*, 1993) and Burkitt's lymphoma (28%) (Kaneko *et al.*, 1996; Preudhomme *et al.*, 1995), suggesting that the frequency of p53 mutations in NHL may be higher than in other hematopoietic malignancies. Activating mutants of Rac1 cooperate with p53 deficiency to promote primary mouse embryonic fibroblast transformation and/or invasion, suggesting a possible functional cooperation between loss of the p53 gene and Rho GTPase-mediated signaling pathways in tumorigenesis.

The human RhoH/TTF gene encodes a 191 amino acid protein belonging to the Rho GTPase family. The C-terminal tail of RhoH, CKIF, represents a typical CAAX motif present in the entire Ras superfamily of small GTP-binding proteins. Proteins containing this motif will be geranylated if the C-terminal amino acid (X) is leucine (L) or phenylalanine (F). This post-translational modification plays a critical role in the localization of Ras and Rho proteins to the plasma membrane (Kinsella *et al.*, 1991). Biochemical studies showed that RhoH is GTPase deficient and remains constitutively in the active, GTP-bound state (Li *et al.*, 2002b). Interestingly, RhoH and RhoE are naturally GTPase deficient due to the amino acid substitutions at key residues that are highly conserved among all Rho GTPases (Li *et al.*, 2002b). This suggests that in contrast to many other family members, regulation of RhoH and RhoE may depend on the level of the protein expressed in the cells rather than guanine nucleotide cycling. Possible mechanisms for regulating RhoH and RhoE activity may include transcriptional, translational, and post-translational processes, which have not been well studied.

Like Rac2, RhoH is expressed only in the hematopoietic lineages, reportedly predominantly in T- and B-cell lines (Dallery-Prudhomme *et al.*, 1997; Li *et al.*, 2002b). Studies in Jurkat cells showed that RhoH expression is transcriptionally regulated upon stimulation with cytokines. Under physiological conditions, RhoH transcripts are also found differentially expressed in murine Th1 and Th2 T-cell subpopulations (Li *et al.*, 2002b), suggesting that RhoH may play a role in differentiation or function in Th1 and Th2 cells. However, these studies have been limited to lymphocytes and are mainly based on cell lines.

Alteration of RhoH expression experimentally affects proliferation and engraftment of hematopoietic progenitor cells (Gu *et al.*, 2005a) and integrin-mediated adhesion in Jurkat cells (Cherry *et al.*, 2004). We and others have determined major physiological functions of RhoH using gene-targeted mice deficient in the RhoH protein. *RhoH*$^{-/-}$ mice demonstrate impaired TCR-mediated thymocyte positive selection and maturation,

resulting in T-cell deficiency (Dorn *et al.*, 2007; Gu *et al.*, 2006). Loss of RhoH leads to defective CD3ζ phosphorylation, impaired translocation of ZAP-70 to the immunological synapse, and reduced activation of ZAP-70-mediated pathways in thymic and peripheral T cells. Furthermore, proteomic analysis demonstrated RhoH to be a component of TCR signaling via TCR-activated ZAP-70 SH2-mediated interaction with immunoreceptor tyrosine-based activation motifs (ITAMs) in RhoH. *In vivo* reconstitution studies showed that RhoH function in thymopoiesis is dependent on phosphorylation of the ITAMs. These findings suggest that RhoH is a critical regulator of thymocyte development and TCR signaling by mediating recruitment and activation of ZAP-70. While a direct relationship has yet to be ascertained, taken together these experiments suggest that alterations in the expression and/or function of RhoH may play a role in lymphoma formation. Clearly RhoH is an important signaling molecule in T-cell development, although its exact role in T-cell receptor signaling remains to be elucidated.

9. Summary and Perspectives

The development of gene-targeted mice deficient in Rac GTPases and the use of knockout mice to study the role of these important molecular switches have contributed to the understanding of the role of Rho GTPase in normal blood cell development and function and, indeed, have led to the delineation of complex functioning of Rho GTPases in primary cells in physiological settings. This is particularly true with regard to the unique functions of different Rac molecules in HSC/P, which has not previously been studied or appreciated because of the lack of specificity of experimental methods relying on activated or dominant negative mutants and cell lines. To summarize, Rac2 deficiency leads to a variety of cellular phenotypes in hematopoietic cells, including abnormalities in cell adhesion, migration, degranulation, and phagocytosis as a consequence of abnormal F-actin assembly. Surprisingly, Rac2 appears to regulate survival in several cell types via activation of Akt pathways. In contrast, Rac1 regulates both overlapping and unique F-actin functions. and these differences appear, at least in neutrophils, to be because of differences in intracellular localization controlled by sequences in the carboxy-terminal tail and a specific region of the protein not previously implicated in Rac function (Filippi *et al.*, 2004). Rac1 regulates HSC/P cell cycle progression. Significantly, Rac1 is critical to stem cell engraftment and Rac2 is a major determinant of HSC/P retention in the marrow cavity. In addition, Cdc42 and RhoA may play an opposite role in HSC/P homing and engraftment. Thus, it is possible that each Rho family member will be utilized differently in these processes.

Overall, these studies implicate Rac, Cdc42, and RhoA as major regulators of HSC/P engraftment and marrow retention and begin to define the ''intracellular signaling profile of the stem cell niche.'' Indeed, these studies suggest that engraftment and mobilization are separable biochemically and imply that these processes are not ''mirror images'' functionally.

These studies have also raised several unanswered questions. They include what the specific effector pathways (such as STAT, PAK, or WAVE) are downstream of individual Rho GTPases critical for stem cell adhesion, engraftment, and retention, as well as for stem cell transformation. Whether the altered cell adhesion and migration properties of HSC/Ps are directly responsible for the engraftment effect and for the survival/proliferation will also need to be dissected. Finally, the specificity of upstream guanine exchange factors coupling to the upstream stimuli of SDF1a, SCF, or integrins in the activation of Rho GTPases will need to be determined in hematopoietic cells.

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